Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 103–106

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Racemization of 6-methoxydihydrosanguinarine in methanol investigated by enantioselective dynamic HPLC

Enqi Wu[a], Sang-Hun Jung[a], Jianbo Chen[a], Young Ho Kim[a], Kyung Lae Park[a], Wonjae Lee[b,*,1], Jong Seong Kang[a,**,1]

[a] College of Pharmacy, Chungnam National University, Yooseong-Ku, Daejeon 305-764, Republic of Korea
[b] College of Pharmacy, Chosun University, Gwangju 501-759, Republic of Korea

ARTICLE INFO

Article history:
Received 19 June 2009
Received in revised form 4 August 2009
Accepted 10 August 2009
Available online 18 August 2009

Keywords:
6-Methoxydihydrosanguinarine
Racemization
Interconversion
Chiral chromatography
Iminium ion intermediate

ABSTRACT

A biologically active benzophenanthridine alkaloid, 6-methoxydihydrosanguinarine (MS), was isolated from Hylomecon plants. Although enantiomers of MS can be separated by chiral HPLC, its isomers rapidly form a racemic mixture in methanol. The rate constants for the racemization of MS enantiomers were 9.20×10^{-4} s^{-1} and 9.95×10^{-4} s^{-1} for (+)-MS and (−)-MS, respectively, as determined by dynamic HPLC and chiral chromatography. This unusually rapid racemization may originate from the formation of a stable iminium ion intermediate, sanguinarine. Therefore, the variety of biological activities exhibited by MS may be attributable to a combination of (+)-MS, (−)-MS, and sanguinarine.

1. Introduction

The biologically active benzophenanthridine alkaloid 6-methoxydihydrosanguinarine (MS) has been isolated from several wild plants, including *Corydalis tashiroi* [1], *Hypecoum leptocarpum* [2], *Fumaria vaillantii* [3], *Fumaria indica* [4], and *Chelidonium majus* [5]. It has shown potential as a chemotherapeutic agent [6,7], nematocide [8,9], acetylcholinesterase inhibitor, and cytotoxic agent against human cancer cell lines [10]. Recently, MS isolated from *Hylomecon hylomeconoides* and *H. vernale* was analyzed by HPLC [11]. *H. hylomeconoides* and *H. vernale*, the only two Hylomecon species growing wild in Korea [12], produce many different benzophenanthridine alkaloids, which exhibit a variety of pharmacological effects.

MS contains a single chiral carbon (Fig. 1), and thus two enantiomers are available. Although unknown, the extent of biological activity is expected to be different for each enantiomer. Efforts to determine the biological activities of isolated MS enantiomers have been confounded by rapid racemization in methanol solution. Knowledge of the interconversion rates between these enantiomers is important for clearly defining the structural characteristics of MS and for establishing its effective utilization. Rate constants for the interconversion of stereoisomers have typically been determined by chromatographic methods such as HPLC [13–16], GC [17–20], CEC [21], or CE [22,23] with the use of approximation functions [24] or computer simulation programs [25–27]. Trapp et al. reviewed the dynamic chromatography and the stopped-flow chromatographic techniques for the determination of enantiomerization and isomerization barriers [28]. In the present study, the interconversion of MS enantiomers during dynamic HPLC and the racemization of MS in methanol solution were evaluated.

2. Experimental

2.1. Preparation of (±)-MS and its enantiomers

Racemic MS was isolated from the roots of *H. hylomeconoides* as reported previously [10] and was identified by ^{1}H NMR, ^{13}C NMR, and mass spectrometry. The purity as confirmed by HPLC was more than 99.5%. Isolated racemic MS was chromatographed on a Chiralcel OD column (4.6 mm × 150 mm, Daicel Chemical Industries Ltd., Tokyo, Japan) with isopropanol–*n*-hexane (5:95, v/v) as the mobile phase and the individual enantiomers were separately collected in an ice bath. Solid (+)-MS and (−)-MS were prepared by evaporating the solvent under a nitrogen stream in an ice bath, and the isolated enantiomers were stored at −70 °C.

* Corresponding author.
** Corresponding author. Tel.: +82 42 821 5928; fax: +82 42 823 6566.
E-mail address: kangjss@cnu.ac.kr (J.S. Kang).
[1] These authors contributed equally to this work.

0731-7085/$ – see front matter.
doi:10.1016/j.jpba.2009.08.014

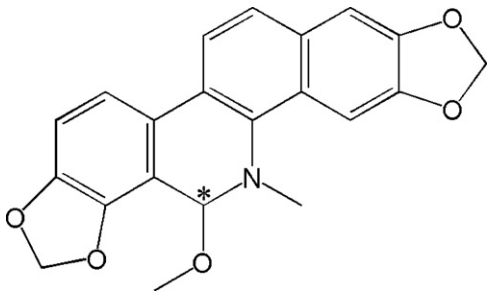

Fig. 1. The structure of 6-methoxydihydrosanguinarine (MS).

2.2. Chromatography

The chromatographic system for chiral analysis consisted of an HPLC Pump 64 (Knauer, Germany), a variable-wavelength UV monitor (Model 655A; Hitachi Co., Japan), and a Chromato-Integrator D-2500 (Hitachi Co.). The enantiomers of MS was separated using two different chromatographic systems. A Chiralcel OJ column (4.6 mm × 150 mm, Daicel Chem.) with methanol as the mobile phase was used for the measurement of interconversion during chromatographic process of racemic MS. A Chiralcel AD column (4.6 mm × 250 mm, Daicel Chem.) with isopropanol–*n*-hexane–diethylamine (20:80:0.1, v/v/v) as the mobile phase was used for the determination of each enantiomers from a pure or partially racemized MS to measure the racemization constant. The separation process was performed at room temperature (25 °C) or at 0 °C by immersing the column in an ice bath. The flow rate was 1.0 ml/min and the effluent was monitored by measuring the optical absorbance at 320 nm.

3. Results and discussion

3.1. Chiral separation of MS enantiomers

Racemic MS was separated into its enantiomers on chiral stationary phases such as Chiralcel AD and OJ. The elution order from the Chiralcel AD column was (+)-MS followed by (−)-MS and was reversed with the OJ column (Fig. 2). The separation factors (α) for the AD and OJ columns were 1.38 and 2.35, respectively, showing baseline separation. Fig. 2a shows that each enantiomer represented 50% of the total peak area of the chromatogram on Chiralcel AD with isopropanol–*n*-hexane–diethylamine as mobile phase, indicating negligible interconversion of enantiomers during chromatography. During enantiomer separation on the OJ column with methanol as mobile phase, a plateau was observed between the two peaks (Fig. 2b); this might have been the result of a slow interconversion of (+)-MS to (−)-MS and vice versa. Presumably, the interconversion occurred on the OJ stationary phase and in methanol during separation. The plateau was eliminated with a reduction of the separation temperature (Fig. 2c). On the Chiralcel OJ with methanol at 0 °C, the peak areas of (+)- and (−)-MS were 42% and 38%, respectively, of the total peak area of the chromatogram, indicating that about 20% of the MS was converted to its opposing enantiomer during separation (Fig. 2c). The conversion rate increased to 72% when the temperature was increased to 25 °C (Fig. 2b).

3.2. Interconversion constants of MS enantiomers

During the HPLC separation of MS, a plateau was observed between the two enantiomer peaks, because the stationary and mobile phases acted as a medium for interconversion. A computer simulation of the experimentally observed elution profile was

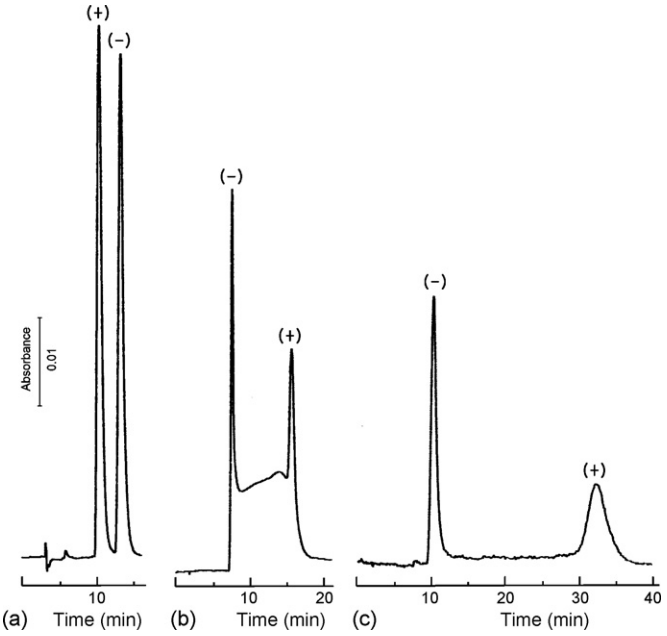

Fig. 2. Chromatograms showing the separation of racemic MS on Chiralcel AD with an isopropanol–*n*-hexane–diethylamine (20:80:0.1, v/v/v) mobile phase at 25 °C (a) and on Chiralcel OJ with methanol at 25 °C (b) and 0 °C (c).

created to obtain overall rate constants for this racemization process during chromatography. The rate constants represent average values of the contributions from processes occurring in both the mobile and stationary phases. Trapp and Schurig [24] suggested an approximation method without computer simulation, described by Eq. (1), to calculate the interconversion rates of racemic mixtures from chromatographic parameters in dynamic chromatography.

$$k_1^{\mathrm{aprx}} = -\frac{1}{t_1}\ln D - \frac{1}{t_1}\ln\left(D + c_1^0\frac{h_\mathrm{p} - e^{\nu_1}}{\sigma_1\sqrt{2\pi}} + c_2^0\frac{h_\mathrm{p}e^{4\nu_2} - e^{\nu_2}}{\sigma_2\sqrt{2\pi}}\right) \quad (1)$$

where

$$h_\mathrm{p} = \frac{c(\bar{t})}{c(t)}, \quad \sigma = w_h/2.355, \quad \nu = -\frac{\Delta t^2}{8\sigma},$$

$$D = \frac{(c_1^0 + c_2^0)}{\Delta t}\left(1 - h_\mathrm{p}\left(0.5 + \frac{1}{\sqrt{2\pi N}}\right)\right)$$

where k_1^{aprx}: approximated forward reaction rate constant of enantiomerization; t_1: retention time of 1st eluted enantiomer; w_h: width of peak at half-height; $c(t)$: height of 1st peak; $c(\bar{t})$: height of plateau; c^0: initial concentration of enantiomers; N: number of theoretical plates.

This equation was applied to the chromatograms in Fig. 2b and c. The parameters obtained from Fig. 2b (25 °C) were t_1 = 8.45 min, t_2 = 17.3 min, h_p = 22.1%, $w_{\mathrm{h}1}$ = 0.333 min, $w_{\mathrm{h}2}$ = 0.889 min, and N = 3566; those from Fig. 2c (0 °C) were t_1 = 10.14 min, t_2 = 32.48 min, h_p = 2.37%, $w_{\mathrm{h}1}$ = 0.80 min, $w_{\mathrm{h}2}$ = 3.04 min, and N = 890. The calculated approximate forward reaction rate constants of racemization for MS on Chiralcel OJ stationary phase with methanol at 25 °C and 0 °C, as calculated by Eq. (1) with the above parameters, were $2.79 \times 10^{-3}\,\mathrm{s}^{-1}$ and $4.50 \times 10^{-4}\,\mathrm{s}^{-1}$, respectively. The rate constant at 25 °C was approximately six times the rate constant at 0 °C. This agrees with the observation that MS enantiomers racemize rapidly at or above room temperature.

3.3. Racemization of MS enantiomers in methanol

The isolated MS enantiomer racemized so rapidly in methanol at room temperature that determination of the rate constant for

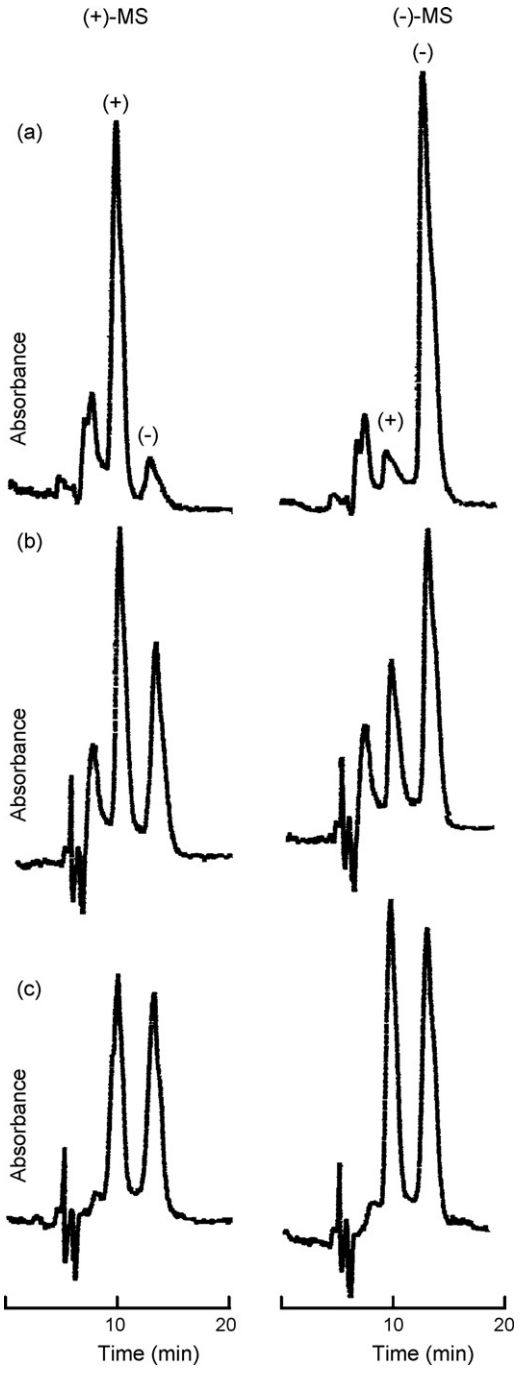

Fig. 3. Racemization profiles for MS enantiomers in methanol. MS enantiomers were dissolved in methanol and allowed to sit for (a) 1 min, (b) 2 min, and (c) 15 min in an ice bath (0 °C) before injection onto the HPLC column. The enantiomers were analyzed by chiral HPLC on Chiralcel AD with a mobile phase of isopropanol–*n*-hexane–diethylamine (20:80:0.1, v/v/v) at room temperature.

racemization was not possible. To determine the rate constant of racemization, each enantiomer was dissolved in ice-cooled methanol and transferred to an ice bath. This point was assigned time = 0 min, although racemization was already in progress. However, racemization was assumed to have stopped during HPLC injection and during the time required to separate the enantiomers on the column. HPLC injections of (+)-MS at known times yielded chromatograms with gradually decreasing proportions of (+)-MS and increasing proportions (−)-MS (Fig. 3, left panel). Analogous experiments were performed with isolated (−)-MS (Fig. 3, right panel). The racemization constant was calculated from the slope of

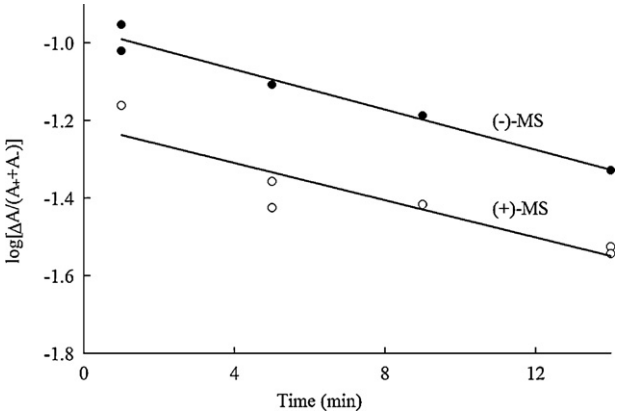

Fig. 4. Log[$\Delta A/(A_+ + A_-)$] as a function of time for (+)-MS (open circle) and (−)-MS (closed circle) in an ice bath.

the semi-log plot of these data according to the equation [15,29]: $2kt/2.3 = \log[\Delta A/(A_+ + A_-)]$, where A_+ and A_- are the peak areas corresponding to (+)-MS and (−)-MS, respectively; k is the interconversion constant; and $2k$ is the racemization constant [30]. The plots showed a linear relationship in both cases, with correlation constants of $R^2 = 0.838$ and 0.970 for (+)- and (−)-MS, respectively. The rate constants of racemization ($2k$), as calculated from the slopes of the lines in Fig. 4, were $9.20 \times 10^{-4}\ s^{-1}$ and $9.95 \times 10^{-4}\ s^{-1}$ for (+)- and (−)-MS, respectively.

3.4. Mechanism for racemization of MS enantiomers via sanguinarine

The racemization of MS can be explained in terms of the equilibrium between MS and the sanguinarine methoxide ion pair. In methanol, the methoxide of MS dissociates to produce the sanguinarine methoxide ion pair, as shown in Fig. 5. The sanguinarine component contains a planar iminium ion, and methanol may add to either side of the iminium group, thereby forming a racemic mixture of MS. This type of rapid racemization is unusual compared with other 2-alkoxypiperidines [31]. 2-Methoxypiperidine hydrochloride (needles, melting point 94–96 °C) has been known to be very stable and could be isolated as a crystallized form in ether [32], although aminal type 2-hydroxypiperidine exists in equilibrium with 1-piperdeine in aqueous solution. Thus enantiomers of 2-alkoxypiperidine can be separated without racemization at room temperature. However, the rapid racemization of MS may be the

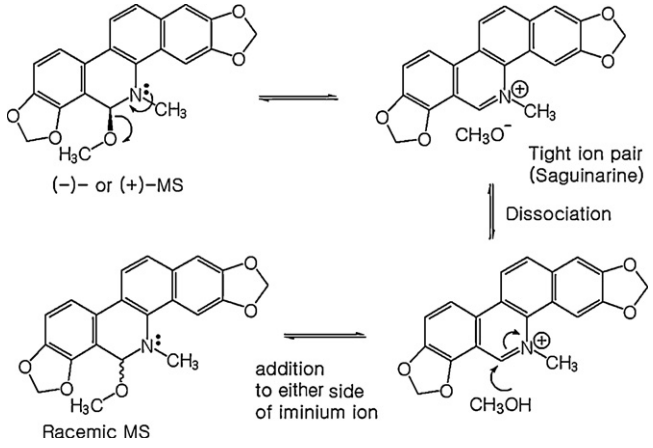

Fig. 5. The proposed mechanism of MS racemization.

result of the relatively high stability of the iminium ion owing to its incorporation into the aromatic structure of sanguinarine.

4. Conclusions

Despite their separation by chiral HPLC techniques, enantiomers of MS rapidly formed a racemic mixture in methanol. The rate constants of racemization were calculated as $9.20 \times 10^{-4}\,s^{-1}$ and $9.95 \times 10^{-4}\,s^{-1}$ for (+)-MS and (−)-MS, respectively. This unusually rapid racemization relative to that of other 2-alkoxypiperidines probably originates from the high stability of the iminium ion intermediate. Therefore, the various observed biological activities of MS may be attributable to a combination of (+)-MS, (−)-MS, and sanguinarine, which all appear during the racemization process of MS.

Acknowledgement

This study was financially supported by the research fund of Chungnam National University in 2008.

References

[1] J.J. Chen, Y.L. Chang, C.M. Teng, W.Y. Lin, Y.C. Chen, I.S. Chen, Planta Med. 67 (2001) 423–427.
[2] G.L. Zhang, G. Ruecker, E. Breitmaier, R. Mayer, Phytochemistry 40 (1995) 1813–1816.
[3] B. Sener, B. Gozler, R.D. Minard, M. Shamma, Phytochemistry 22 (1983) 2073–2075.
[4] V.B. Pandey, A.B. Ray, B. Dasgupta, Phytochemistry 18 (1979) 695–696.
[5] G. Kadan, T. Gozler, M. Shamma, J. Nat. Prod. 53 (1990) 531–532.
[6] H.Q. Yin, Y.H. Kim, C.K. Moon, B.H. Lee, Biochem. Pharmacol. 70 (2005) 242–248.
[7] Y.J. Lee, H.Q. Yin, Y.H. Kim, G.Y. Li, B.H. Lee, Arch. Pharm. Res. 27 (2004) 1253–1257.
[8] T. Satou, N. Akao, R. Matsuhashi, K. Koike, K. Fujita, T. Nikaido, Biol. Pharm. Bull. 25 (2002) 1651–1654.
[9] R. Matsuhashi, T. Satou, K. Koike, A. Yokosuka, Y. Mimaki, Y. Sashida, T. Nikaido, Planta Med. 68 (2002) 169–171.
[10] Y.S. Cho, Doctor's Thesis. Chungnam National University, 2001, p. 50.
[11] J.S. Kang, P.H. Long, H.M. Lim, Y.H. Kim, B. Gottfried, Arch. Pharm. Res. 26 (2003) 114–119.
[12] Y.N. Lee, Flora of Korea, Kyohaksa, Seoul, 1996, pp. 237–238.
[13] O. Trapp, G. Trapp, V. Schurig, J. Biochem. Biophys. Methods 54 (2002) 301–313.
[14] O. Trapp, S. Caccamese, C. Schmidt, V. Bohmer, V. Schurig, Tetrahedron Asymmetry 12 (2001) 1395–1398.
[15] S. Caccamese, G. Principato, Tetrahedron Asymmetry 9 (1998) 2939–2945.
[16] K. Cabrera, M. Jung, M. Fluck, V. Schurig, J. Chromatogr. A 731 (1996) 315–321.
[17] O. Trapp, V. Schurig, Chirality 14 (2002) 465–470.
[18] V. Schurig, M. Jung, M. Schleimer, F.G. Klaerner, Chem. Ber. 125 (1992) 1301–1303.
[19] O. Trapp, G. Schoetz, V. Schurig, J. Pharm. Biomed. Anal. 27 (2002) 497–505.
[20] O. Trapp, V. Schurig, J. Am. Chem. Soc. 122 (2000) 1424–1430.
[21] G. Schoetz, O. Trapp, V. Schurig, Electrophoresis 22 (2001) 3185–3190.
[22] G. Schoetz, O. Trapp, V. Schurig, J. Capill. Electrophor. Microchip Technol. 6 (1999) 169–175.
[23] S. Gabriele, O. Trapp, V. Schurig, Enantiomer 5 (2000) 391–396.
[24] O. Trapp, V.J. Schurig, J. Chromatogr. A 911 (2001) 167–175.
[25] O. Trapp, Chirality 18 (2006) 489–497.
[26] O. Trapp, V. Schurig, Comput. Chem. 25 (2001) 187–195.
[27] O. Trapp, Anal. Chem. 78 (2006) 189–198.
[28] O. Trapp, G. Schoetz, V. Schurig, Chirality 13 (2001) 403–414.
[29] N. Fujii, Y. Momose, N. Ishii, M. Takita, M. Akaboshi, M. Kodama, Mech. Age. Dev. 107 (1999) 347–358.
[30] E.L. Eliel, S.H. Wilen, L.N. Mander (Eds.), Stereochemistry of Organic Compounds, John Wiley & Sons, New York, 1994, p. 426.
[31] J. Chen, C.L. Cantrell, H. Shang, M.G. Rojas, J. Agric. Food Chem. 57 (2009) 3128–3133.
[32] C. Schopf, H. Koop, G. Werner, F. Braun, Franz, Chem. Ber. 93 (1960) 2457–2467.