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Chiral HPLC studies on chemical behavior of 6-methoxydihydrosanguinarine in alcoholic solvent system

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ABSTRACT

All the enantiomers of methoxydihydrosanguinarine (MS), ethoxydihydrosanguinarine (ES) and isopropoxydihydrosanguinarine (PS) were separated by chiral HPLC. They were further identified by comparing the retention times of authentic standards as well as LC–MS. Interestingly, the approximately same conversion rates for the formation MS from ES or PS and the slower conversion of MS in isopropanol compared to ethanol demonstrated two step mechanism in the reaction of alkoxysanguinarine in alcohols, which is composed of the initial formation of sanguinarine as a planar intermediate and the addition of alcohol to intermediate as possible rate limiting step. Thus, sanguinarine has a pivotal role in the chemical behavior of alkoxysanguinarine in alcoholic solvents. Such possible variation of the structure of sanguinarine may be the source of its diverse biological activities.

1. Introduction

Sanguinarine (13-methyl-[1,3]benzodioxolo[5,6-*c*]-1,3-dioxolo[4,5-*i*]phenanthridinium; Fig. 1), which is derived from the root of *Sanguinaria canadendid* and other poppy fumaria species, is a benzophenanthridine alkaloid and a structural homologue of chelerythrine [1,2]. Many sanguinarine derivatives were isolated from the extract of *Hylomecon hylomeconoides* roots and have been reported for their diverse biological activities such as chemotherapeutic agent [3,4], nematocide [5,6], acetylcholinesterase inhibitor, and cytotoxic agent against human cancer cell lines [7]. It has also been reported [8,9] that the sanguinarine derivatives are easily prepared by treatment of sanguinarine free base with an appropriate alcohol. 6-Methoxydihydrosanguinarine (MS), isolated from *H. hylomeconoides,* might be the artifact formed in the methanol extraction. Recently, MS isolated from *H. hylomeconoides* and *Hylomecon vernale* was analyzed by HPLC [10]. *H. hylomeconoides* and *H. vernale*, the only two *Hylomecon* species growing wild in Korea [11] 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. The efforts to determine biological activities of the isolated MS enantiomers have been confounded by rapid racemization. The knowledge of the interconversion rates between these enantiomers in various medium is important for clearly defining the structural characteristics of MS and for establishing its effective utilization.

The rate constants for the interconversion of stereoisomers have typically been determined by chromatographic methods such as HPLC [12–20], GC [21–24], CEC [25], or CE [26,27] with the use of approximation functions [28] or computer simulation programs [29–31]. Trapp et al. reviewed the dynamic chromatography and the stopped-flow chromatographic techniques for the determination of enantiomerization and isomerization barriers [32]. Recently, our group has reported the separation of MS enantiomers using dynamic HPLC and their racemization in methanol solution. During our studies we observed that despite the separation of these isomers by chiral HPLC technique, enantiomers of MS rapidly formed a racemic mixture in methanol [33]. In addition, we elaborated that the unusually rapid racemization of MS in methanol was probably originated due to the formation of highly stable intermediate i.e. iminium ion. We further, concluded that the appearance of various biological activities of MS may be due to the combination of (+)-MS, (−)-MS, and sanguinarine, which all appeared during the racemization process of MS. Therefore, in the current article we studied the effect of different alcoholic solvent systems on MS to elaborate its structural characteristics.

2. Experimental

2.1. General

Methanol (J.T. Baker, NJ, USA), isopropanol (Merck, Germany) and n-hexane (Fisher Chem., UK) used in this work were of

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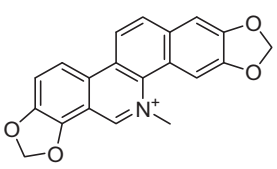
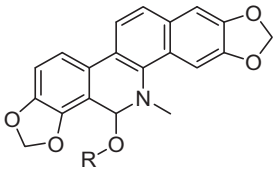


1, Sanguinarine



2a, R = CH_3, 6-Methoxydihydrosanguinarine (MS)
2b, R = CH_2CH_3, 6-Ethoxydihydrosanguinarine (ES)
2c, R = $CH(CH_3)_2$, 6-Isopropoxydihydrosanguinarine (PS)

Fig. 1. Derivatives of sanguinarine.

HPLC grade and other reagents were of analytical grade. Sanguinarine was purchased from Sigma–Aldrich (MI, USA). The chromatographic system for chiral analysis consisted of LC-10AD pump, SCL-10AD system controller and SPD-10AVP diode array detector (Shimadzu, Kyoto, Japan). The chiral analysis was carried out on Chiralcel OD-H (4.6 mm × 250 mm) and Chiralcel OD (4.6 mm × 50 mm) columns from Daicel Co. (Japan).

2.2. Preparation of authentic stereoisomers of MS, ES and PS

Racemic MS was isolated from roots of *H. hylomeconoides* as reported previously [7] and identified by ^{1}H NMR, ^{13}C NMR and MS. The purity confirmed by HPLC was more than 99.5%. Same compound could also be prepared by dissolving sanguinarine in methanol. (±)-MS was treated on Chiralcel OD-H column and enantiomers were separated with isopropanol-n-hexane (20:80, v/v) by the flow rate of 1 mL/min. The individual enantiomers were separately collected on ice bath. Authentic enantiomers were prepared by evaporating the solvent under nitrogen stream on ice bath and stored at −70 °C. Authentic enantiomers of ES and PS were prepared by the same method.

2.3. Chromatographic separation and identification of enantiomers of MS, ES and PS

The chiral separation of mixture of MS, ES and PS was performed using chiral columns. The eluents for Chiralcel OD-H column was isopropanol-n-hexane (20:80, v/v) with a flow rate of 1 mL/min. The system was operated generally at room temperature but sometimes it was necessary to reduce the column temperature, so the column was immersed in ice bath. Detection was carried out at UV 320 nm. LC–MS spectra were obtained with APCI positive mode at 4.5 kV (150 °C), nebulizer gas flow rate of 2.0 L/min, CDL voltage of −60 V, deflector voltage of 20 V, and CDL temperature of 230 °C as shown in Fig. 2.

2.4. Measurement of racemization constant of ES and PS enantiomers

The ES and PS enantiomers were separately collected on ice bath. Racemization started when 1 mg of (+)/(−)-ES and (+)/(−)-PS were dissolved in 10 mL of ethanol and isopropanol, respectively, and the solutions were stored at 25 °C for limited times. The enantiomers were analyzed by chiral HPLC on Chiralcel OD column with isopropanol-n-hexane (20:80, v/v) as mobile phase at 1 mL/min of flow rate.

2.5. Conversion of racemic MS to PS and ES

One mg of (±)-MS was dissolved in 10 mL of isopropanol and stood at 25 °C. After different time periods as indicated in Fig. 3, 10 μL of solution was injected to HPLC and the remaining MS and PS enantiomers were analyzed. The same experiments were carried out in ethanol at 25 °C and 0 °C. For the separation of MS, ES and PS enantiomers Chiralcel OD-H column was used, but Chiralcel OD column was used for the analysis of ethanol solution of MS at 25 °C as shown in Fig. 3.

2.6. Conversion of PS or ES to MS

One mg of (±)-ES and (±)-PS was separately dissolved in 10 mL of methanol and kept for 1 and 12 min at 25 °C. The produced MS were analyzed on Chiralcel OD-H column with isopropanol-n-hexane (20:80, v/v) as shown in Fig. 4.

3. Results and discussion

3.1. Chiral separation and identification of compounds

The separation of a mixture of isomers for MS, ES and PS has been carried out using Chiralcel OD-H column. The elution order was (+)-PS, (+)-ES, (−)-PS, (+)-MS, (−)-ES, and (−)-MS as shown in Fig. 2a. The isomers were identified by comparing them with the retention times of authentic standards and they were further identified by LC–MS as shown in Fig. 2b. (+)-PS was identified with m/z 392 $[M+H]^+$ shown in Fig. 2b(1), (+)-ES (Fig. 2b(2)) with m/z 378 $[M+H]^+$, (−)-PS (Fig. 2b(3)) with m/z 392 $[M+H]^+$, (+)-MS (Fig. 2b(4)) with m/z 364 $[M+H]^+$, (−)-ES (Fig. 2b(5)) with m/z 378 $[M+H]^+$, and (−)-MS (Fig. 2b(6)) with m/z 364 $[M+H]^+$. All mass spectra showed the base peak m/z 332 due to the loss of methoxy, ethoxy and isopropoxy moiety from MS, ES and PS, respectively.

3.2. Rate constants for the conversion of MS to ES and PS

The rate constant of the racemization (10.04 h^{-1}) for MS at 25 °C was determined as reported in our earlier studies [33]. The rate constants for the racemization of ES and PS determined by the chiral separation of (+)/(−)-ES and (+)/(−)-PS on Chiralcel OD column were 2.38 h^{-1} and 4.55×10^{-2} h^{-1}, respectively. The rate of racemization sequentially decreased in order of MS, ES and PS possibly due to the increasing size or the decreasing nucleophilicity of methanol, ethanol and isopropanol as indicated with dielectric constants (methanol $\varepsilon = 33$, ethanol $\varepsilon = 24$ and isopropanol $\varepsilon = 18$). These properties of solvents are reflected on the different conversion rates from MS to ES and from MS to PS as shown below.

The racemic MS was dissolved in isopropanol and the reaction mixture was kept for various time periods at 25 °C, the amount of MS and PS was measured by chiral HPLC in the course of time as shown in Fig. 3a. The same experiment was also carried out in ethanol at 25 °C (Fig. 3b) and 0 °C (Fig. 3c).

The conversion constant was calculated from the slope of the semi-log plot of data according to the equation [14,34]. $2kt/2.3 = \log[\Delta A/(A_P + A_R)]$, where A_P and A_R are the peak areas corresponding to produced and reduced species, respectively. ΔA is the difference between $A_P - A_R$ and k is the conversion constant. The rate constant observed for conversion of MS to PS at 25 °C

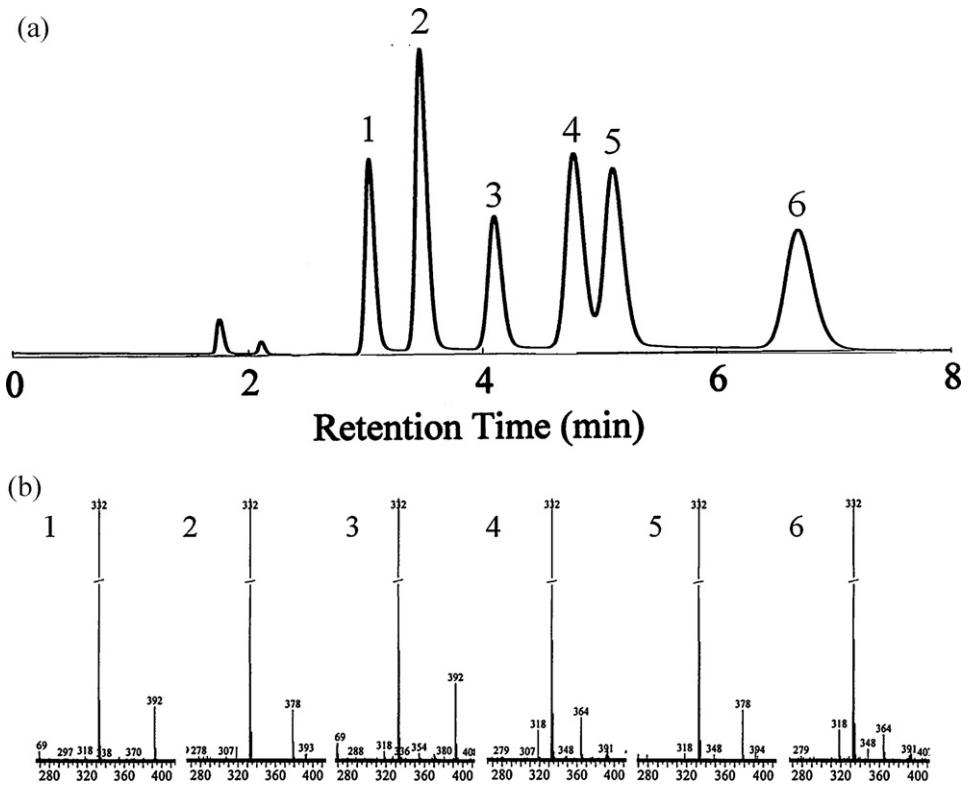


Fig. 2. (a) Separation of the enantiomers of MS, ES and PS on Chiralcel OD-H with isopropanol-n-hexane (20:80, v/v) and (b) LC–MS spectra of separated peaks. MS conditions: Ionization; APCI positive with 4.5 kV (150 °C), nebulizer gas flow rate; 2.0 L/min, CDL voltage; −60 V, deflector voltage; 20 V, CDL temp.; 230 °C. Peak identification: 1. (+)-PS, 2. (+)-ES, 3. (−)-PS, 4. (+)-MS, 5. (−)-ES, 6. (−)-MS.

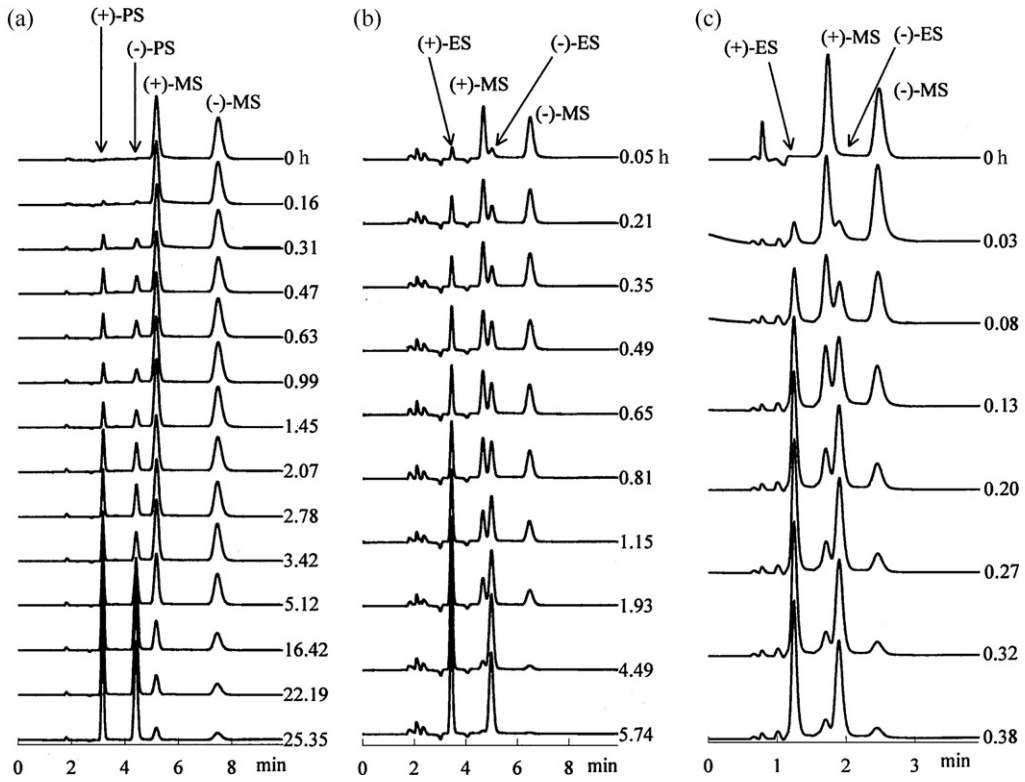


Fig. 3. Conversion of MS to PS in isopropanol at 25 °C (a) and to ES in ethanol at 0 °C (b) and at 25 °C (c). The time allowed to stay at given temperature was indicated at the end of each chromatogram in hours. The enantiomers were analyzed by chiral HPLC on Chiralcel OD-H (a and b) and Chiralcel OD (c).

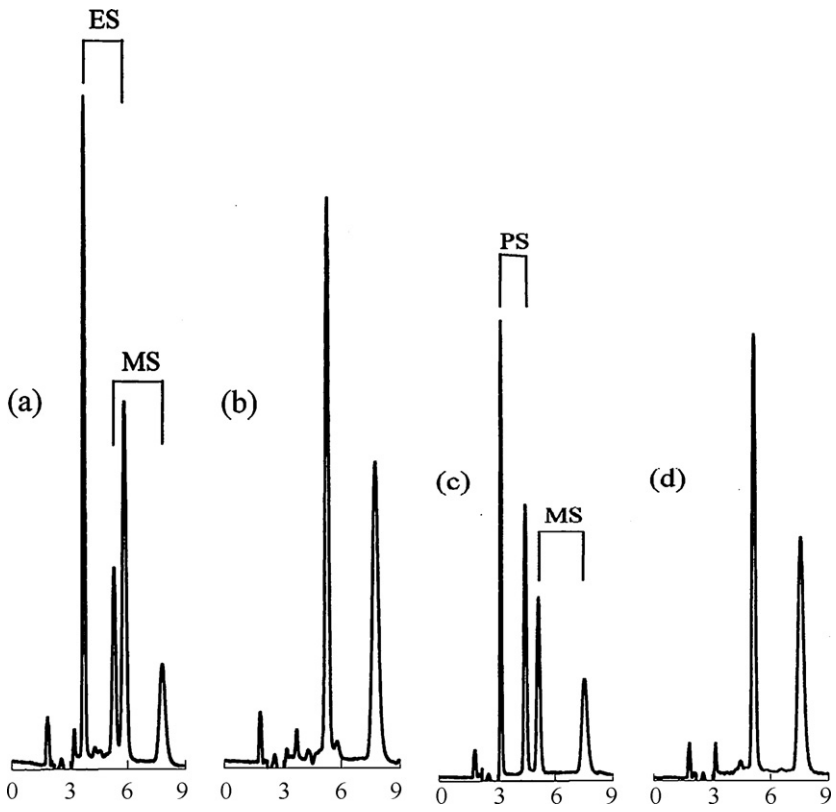


Fig. 4. Formation of MS in methanol from ES (a and b) and PS (c and d) for 1 min (a and c) and 12 min (b and d).

in isopropanol was $6.32 \times 10^{-2}\ h^{-1}$, and that to ES in ethanol was $4.43\ h^{-1}$. This concludes that MS is converted to ES in ethanol about 70 times faster than PS in isopropanol. The rate constants observed for conversion of MS to ES in ethanol at 0 °C was $4.44 \times 10^{-1}\ h^{-1}$. The rates of formation of ES and PS were dependent upon alcoholic solvent, time and temperature. Interestingly, it took about 2 days for complete conversion of MS to PS in isopropanol as shown in Fig. 3a, but in ethanol MS was converted to ES within 5–7 h at 25 °C (Fig. 3b). These results showed that the replacement of methoxy group of MS with ethoxy group was faster than with isopropoxy group. Thisimplies that the formation of ethanol–sanguinarine transition state is easier than the isopropanol–sanguinarine transition state, maybe due to the larger size and low dielectric constant of isopropanol.

However, when racemic ES and PS were dissolved in methanol, 99% and 98% of initial contents, respectively, were converted into MS in 12 min. The rate constants for the conversion of ES and PS to MS were $16.6\ h^{-1}$ and $16.8\ h^{-1}$, respectively, as shown in Fig. 4. The rates of formation of MS from ES or PS are nearly same. Thus this indicates that the fast formation of common intermediate from the both reactions and the addition of methanol to this intermediate is the rate determining step.

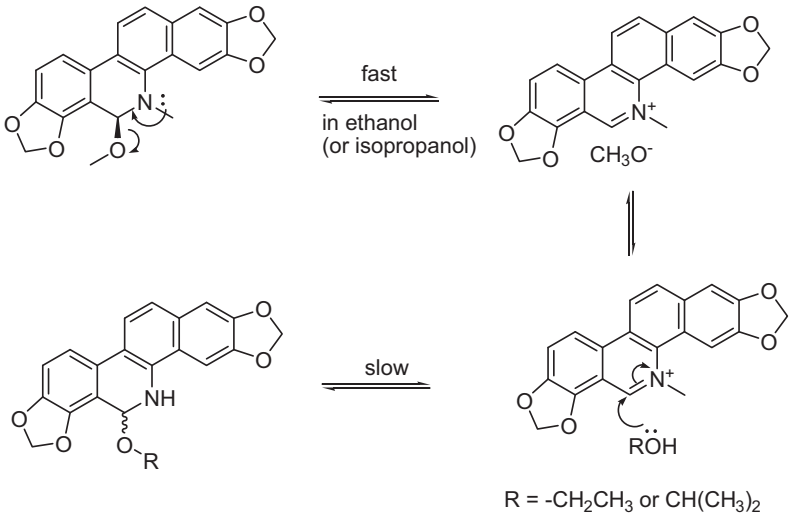


Fig. 5. Mechanism for the conversion of MS in alcoholic solvents via sanguinarine intermediate.

3.3. Conversion mechanism of MS to ES and PS

As we have discussed earlier [33] the racemization of MS can occur due to the equilibrium between MS and the sanguinarine methoxide ion pair. However the rate limiting step was not determined. Similarly, the conversion of MS to ES and PS in ethanol and isopropanol, respectively, can occur via planar sanguinarine, which contributed the racemized conversion of MS to ES or PS as shown in Fig. 5. The same rate for the formation of MS from ES or PS proves that these conversions undergo through a common intermediate, sanguinarine. The seventy times slower conversion of MS to PS than that of MS to ES implies that the formation of ethanol–sanguinarine transition state is easier than the isopropanol–sanguinarine transition state due to the larger size or the lower nucleophilicity of isopropanol and thus the rate limiting step should be the addition of alcohol to sanguinarine rather than formation of sanguinarine from alkoxysanguinarine. Thus the formation of highly stable iminium ion intermediate owing to its incorporation into the aromatic structure of sanguinarine leads to this characteristic interconversion.

4. Conclusions

All the enantiomers of MS, ES and PS were separated by chiral HPLC. They were further identified by comparing the retention times of authentic standards as well as LC–MS. Interestingly, the same conversion rates for the formation MS from ES or PS and the slower conversion of MS in isopropanol compared to ethanol demonstrated two step mechanism in the reaction of alkoxysanguinarine in alcohols, which is composed of the initial formation of sanguinarine as a planar intermediate and the addition of alcohol to intermediate as possible rate limiting step. Thus, sanguinarine has a pivotal role in the chemical behavior of alkoxysanguinarine in alcoholic solvents. Such possible variation of the structure of sanguinarine may be the source of its diverse biological activities.

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References

[1] M. Shamma, H. Guinaudeau, Aporphinoid alkaloids, Nat. Prod. Rep. 3 (1986) 345–351.
[2] J.M. Herbert, J.M. Augereau, J. Gleye, J.P. Maffrand, Chelerythrine is a potent and specific inhibitor of protein kinase C, Biochem. Biophys. Res. Commun. 172 (1990) 993–999.
[3] H.Q. Yin, Y.H. Kim, C.K. Moon, B.H. Lee, Reactive oxygen species-mediated induction of apoptosis by a plant alkaloid 6-methoxydihydrosanguinarine in HepG2 cells, Biochem. Pharmacol. 70 (2005) 242–248.
[4] Y.J. Lee, H.Q. Yin, Y.H. Kim, G.Y. Li, B.H. Lee, Apoptosis inducing effects of 6-methoxydihydrosanguinarine in HT29 colon carcinoma cells, Arch. Pharm. Res. 27 (2004) 1253–1257.
[5] T. Satou, N. Akao, R. Matsuhashi, K. Koike, K. Fujita, T. Nikaido, Inhibitory effect of isoquinoline alkaloids on movement of second-stage larvae of *Toxocara canis*, Biol. Pharm. Bull. 25 (2002) 1651–1654.
[6] R. Matsuhashi, T. Satou, K. Koike, A. Yokosuka, Y. Mimaki, Y. Sashida, T. Nikaido, Nematocidal activity of isoquinoline alkaloids against a species of diplogastridae, Planta Med. 68 (2002) 169–171.
[7] Y.S. Cho, Doctor's Thesis, Chungnam National University, 2001, p. 50.
[8] Y. Konda, Y. harigaya, M. Onda, Studies on the constituents of *Bocconia cordata*. III. Structure elucidation of bocconine by means of nuclear magnetic resonance spectroscopic studies, J. Heterocyclic Chem. 23 (1986) 877–879.
[9] R.D. Williams, B.E. Ellis, Alkaloids from *Agrobacterium rhizogenes*-transformed *Papaver somniferum* cultures, Phytochemistry 32 (1993) 719–723.
[10] J.S. Kang, P.H. Long, H.M. Lim, Y.H. Kim, G. Blaschke, Achiral and chiral determination of benzophenanthridine alkaloids from methanol extracts of hylomecon species by high performance liquid chromatography, Arch. Pharm. Res. 26 (2003) 114–119.
[11] Y.N. Lee, Flora of Korea, Kyohaksa, Seoul, 1996, p. 237.
[12] O. Trapp, G. Trapp, V. Schurig, Direct calculation and computer simulation of the enantiomerization barrier of oxazepam in dynamic HPLC experiments—a comparative study, J. Biochem. Biophys. Methods 54 (2002) 301–313.
[13] O. Trapp, S. Caccamese, C. Schmidt, V. Bohmer, V. Schurig, Enantiomerization of an inherently chiral resorcarene derivative: determination of the interconversion barrier by computer simulation of the dynamic HPLC experiment, Tetrahedron: Asymmetry 12 (2001) 1395–1398.
[14] S. Caccamese, G. Principato, Resolution of the enantiomers of tetrahydrozoline by chiral HPLC. The racemization of the enantiomers via an imine–enamine tautomerism, Tetrahedron: Asymmetry 9 (1998) 2939–2945.
[15] K. Cabrera, M. Jung, M. Fluck, V. Schurig, Determination of enantiomerization barriers by computer simulation of experimental elution profiles obtained by high-performance liquid chromatography on a chiral stationary phase, J. Chromatogr. A 731 (1996) 315–321.
[16] J. Veciana, M.I. Crespo, Dynamic HPLC: a method for determining rate constants, energy barriers, and equilibrium constants of molecular dynamic processes, Angew. Chem. Int. Ed. 30 (1991) 74–77.
[17] F. Gasparrini, D. Misiti, M. Pierini, C. Villani, Enantiomerization barriers by dynamic HPLC. Stationary phase effects, Tetrahedron: Asymmetry 8 (1997) 2069–2073.
[18] C. Wolf, Stereolabile chiral compounds: analysis by dynamic chromatography and stopped-flow methods, Chem. Soc. Rev. 34 (2005) 595–608.
[19] I. D'Acquarica, F. Gasparrini, M. Pierini, C. Villani, G. Zappia, Dynamic HPLC on chiral stationary phases: a powerful tool for the investigation of stereomutation processes, J. Sep. Sci. 29 (2006) 1508–1516.
[20] J. Krupcik, J. Mydlova, P. Majek, P. Simon, D.W. Armstrong, Methods for studying reaction kinetics in gas chromatography, exemplified by using the 1-chloro-2,2-dimethylaziridine interconversion reaction, J. Chromatogr. A 1186 (2008) 144–160.
[21] O. Trapp, V. Schurig, Novel direct access to enantiomerization barriers from peak profiles in enantioselective dynamic chromatography: enantiomerization of dialkyl-1,3-allenedicarboxylates, Chirality 14 (2002) 465–470.
[22] V. Schurig, M. Jung, M. Schleimer, F.G. Klaerner, Investigation of the enantiomerization barrier of homofuran by computer simulation of interconversion profiles obtained by complexation gas chromatography, Chem. Ber. 125 (1992) 1301–1303.
[23] O. Trapp, G. Schoetz, V. Schurig, Stereointegrity of thalidomide: gas-chromatographic determination of the enantiomerization barrier, J. Pharm. Biomed. Anal. 27 (2002) 497–505.
[24] O. Trapp, V. Schurig, Stereointegrity of Tröger's base: gas-chromatographic determination of the enantiomerization barrier, J. Am. Chem. Soc. 122 (2000) 1424–1430.
[25] G. Schoetz, O. Trapp, V. Schurig, Determination of the enantiomerization barrier of thalidomide by dynamic electrokinetic chromatography, Electrophoresis 22 (2001) 3185–3190.
[26] G. Schoetz, O. Trapp, V.J. Schurig, Determination of the enantiomerization barrier of chlorthalidone by dynamic electrokinetic chromatography and computer simulation, J. Capill. Electrophor. Microchip Technol. 6 (1999) 169–175.
[27] S. Gabriele, O. Trapp, V. Schurig, Determination of the enantiomerization barrier of oxazepam by dynamic micellar electrokinetic chromatography – comparison of experiment and simulation with ChromWin 99, Enantiomer 5 (2000) 391–396.
[28] O. Trapp, V.J. Schurig, Approximation function for the direct calculation of rate constants and Gibbs activation energies of enantiomerization of racemic mixtures from chromatographic parameters in dynamic chromatography, J. Chromatogr. A 911 (2001) 167–175.
[29] O. Trapp, Fast and precise access to enantiomerization rate constants in dynamic chromatography, Chirality 18 (2006) 489–497.
[30] O. Trapp, V. Schurig, ChromWin – a computer program for the determination of enantiomerization barriers in dynamic chromatography, Comput. Chem. 25 (2001) 187–195.
[31] O. Trapp, A unified equation for fast and precise access to rate constants of first order reactions in dynamic and on-column reaction chromatograph, Anal. Chem. 78 (2006) 189–198.
[32] O. Trapp, G. Schoetz, V. Schurig, Review: determination of enantiomerization barriers by dynamic and stopped-flow chromatographic methods, Chirality 13 (2001) 403–414.
[33] E. Wu, S.-H. Jung, J. Chen, Y.H. Kim, K.L. Park, W. Lee, J.S. Kang, Racemization of 6-methoxydihydrosanguinarine in methanol investigated by enantioselective dynamic HPLC, J. Pharm. Biomed. Anal. 51 (2010) 103–106.
[34] N. Fujii, Y. Momose, N. Ishii, M. Takita, M. Akaboshi, M. Kodama, Mech. Ageing Dev. 107 (1999) 347–358.