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Determination of the (*R*)- and (*S*)-enantiomers of salsolinol and N-methylsalsolinol by use of a chiral high-performance liquid chromatographic column

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Abstract

A new method for the quantitative determination of the enantiomers of salsolinol and N-methylsalsolinol, biologically important alkaloids, is reported. The enantiomers were completely separated without derivatization, using a cyclodextrin-modified silica gel column with an HPLC-electrochemical detection system. The HPLC conditions were examined for the best resolution. The method was sensitive enough to detect salsolinol and N-methylsalsolinol at a concentration of less than 0.1 pmol per injection. In the product of the Pictet–Spengler reaction of acetaldehyde with dopamine or epinine, almost equimolar (*R*)- and (*S*)-enantiomers of salsolinol and N-methylsalsolinol were detected. Preliminary results indicate that the (*R*)-enantiomer of both isoquinoline derivatives predominate in the human brain.

1. Introduction

Salsolinol (Sal), 1 - methyl - 6,7 - dihydroxy - 1,2,3,4 - tetrahydroisoquinoline, is a dopamine-derived alkaloid, which was found for the first time in the urine of parkinsonian patients treated with L-DOPA [1]. Later on it was identified in the urine and cerebrospinal fluid of healthy human controls [2–4], as well as in the brains of non-alcoholic subjects [5]. Recently, N-methylated derivatives of Sal and norsalsolinol (6,7 - dihydroxy - 1,2,3,4 - tetrahydroisoquinoline) were also detected in human brains by GC–MS [6]. It has been suggested that the occurrence of these alkaloids in the brain may be involved in some neurological and psychiatric disorders [7,8]. Sal has also been found in some foods and beverages, such as dried banana and port wine [9,10], suggesting that the intake of Sal might affect Sal levels in tissues [11]. However, Sal cannot be transported into the brain through the blood–brain barrier, so that the Sal detected in the brain requires in situ generation [12]. Sal and N-methylsalsolinol (NMSal, 1,2-dimethyl-6,7-

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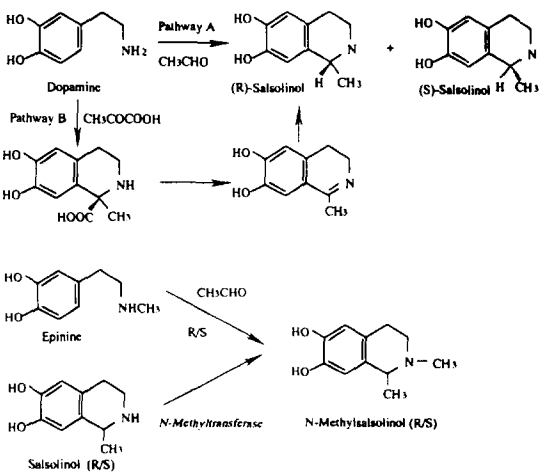

Fig. 1. Biosynthesis pathway of Sal and its derivatives.

dihydroxy-1,2,3,4-tetrahydroisoquinoline) have an asymmetric center at C1 and exist as (*R*)- and (*S*)-enantiomers. As shown in Fig. 1, one biosynthesis pathway of salsolinol is the non-enzymatic Pictet–Spengler condensation of dopamine with acetaldehyde to yield the racemic mixture of both enantiomers (Pathway A). Another pathway (Pathway B) is condensation of dopamine with pyruvic acid, followed by enzymatic decarboxylation and reduction. If the reduction occurs stereospecifically, only (*R*)-Sal would be produced [7,13]. More recently it is suggested that (*R*)-Sal might be formed enzymatically by stereospecific condensation of dopamine with pyruvic acid [Naoi et al., in prep.]. It has also been reported that N-methyldopamine (epinine) occurs in the parkinsonian and normal human brain [15]. Although the enzymatic N-methylation of Sal into NMSal was confirmed by in vivo microdialysis in the rat brain [16], the in vitro experiment suggests that it is also synthesized from epinine and acetaldehyde by the Pictet–Spengler reaction [17].

There is evidence that the (*R*)- and (*S*)-enantiomers of Sal have different biological characteristics. (*S*)-Sal was found to be a more effective inhibitor of dopamine accumulation into rat brain slices than the (*R*)-enantiomer [18]. Both (*R*)- and (*S*)-Sal inhibit type A of monoamine oxidase [monoamine: oxygen oxidoreductase (deaminating), EC 1.4.3.4] competitively, with (*R*)-Sal being a more potent inhibitor than its enantiomeric counterpart [19]. The effect of (*R*)- and (*S*)-Sal on the kinetic properties of tyrosine hydroxylase [tyrosine, tetrahydrobiopterin: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] was also examined, and the asymmetric center of Sal at C1 was found to play an important role in the affinity to L-tyrosine [20]. Recently by injection of these catechol isoquinolines into the rat brain, (*R*)-NMSal caused behavioral changes similar to those seen in patients with Parkinson's disease, whereas (*R*)- and (*S*)-Sal, and (*S*)-NMSal, did not [21]. When using human dopaminergic neuroblastoma SH-SY5Y cells, only (*R*)-NMSal was transported by the dopamine uptake system [22]. These results indicate that the stereochemical structure of these alkaloids is involved in their biological activities, especially in the brain. Therefore, the enantiomeric composition of salsolinol and N-methylsalsolinol should be quantitatively determined in biological samples, such as brain tissue.

The identification of the two enantiomers of Sal was first achieved by GC and nitrogen–phosphorus detection after methylation with diazomethane and then derivatization to the diastereoisomers with N-trifluoroacetyl-L-prolyl chloride [23]. It was found that (*R*)-Sal at least predominates in human urine from healthy volunteers. An HPLC method was developed with the same derivatization method, and the (*R*)/(*S*) ratio of Sal was found to be almost one in bananas [14]. Then, this method was modified by using a chiral derivatizing agent, (*S*)-1-(1-naphthyl)ethyl isothiocyanate, and a good resolution of the two diastereoisomers was obtained [24]. Using this method, it was demonstrated that a minor but definite amount of (*S*)-enantiomer was detected in urine of some healthy volunteers [11]. In addition, both (*R*)- and (*S*)-enantiomers of salsolinol were detected in human urine of alcoholics and of parkinsonian patients after L-DOPA treatment [25,26]. The results suggest that (*S*)-Sal might be synthesized in humans by the Pictet–Spengler reaction only with elevated concentration of aldehyde and dopamine. In all

the above-mentioned papers, the enantiomers of Sal must first be derivatized to the diastereoisomers. These methods were not sensitive enough to detect the enantiomers in human brain samples. The enantiomeric composition of salsolinols in the human brain remains to be determined. This article reports a new HPLC-electrochemical detection (ECD) method for the quantification of the enantiomers of Sal and NMSal without derivatization by using a cyclodextrin column.

2. Experimental

2.1. Chemicals and reagents

Both (*R*)- and (*S*)-enantiomers of Sal and NMSal were synthesized according to Teitel et al. [27]. Dopamine and epinine were purchased from Sigma (St. Louis, MO, USA). All chemicals were of analytical grade and organic solvents were HPLC grade from Nacalai Tesque (Kyoto, Japan).

2.2. Synthesis of Sal and NMSal

Sal was produced by the Pictet–Spengler condensation of dopamine with acetaldehyde. The reaction was carried out with 500 nmol of acetaldehyde and 10–40 nmol of dopamine in 1 ml of 0.1 m*M* Tris-HCl buffer, pH 7.4. After incubation at 37°C for 3 h, the reaction mixture was centrifuged at 22 000 *g* for 10 min. The supernatant was filtered through a Millipore HV filter (pore size 0.45 μm). NMSal was synthesized from epinine and acetaldehyde under the same conditions. The enantiomeric compositions of Sal and NMSal were quantitatively determined by HPLC.

2.3. Preparation of human brain sample

Grey matter of the human brain was dissected and weighed (ca. 1.1 g wet weight), then ten volumes of distilled water (ca. 11 ml) was added. The mixture was sonicated in a Branson sonicator (Danbury, CT, USA) for 1 min with 50% duty cycle, and stored at −80°C. An aliquot (800 μl) of the homogenate was mixed with 100 μl of 1 *M* perchloric acid containing 0.4 m*M* sodium metabisulfite and 0.1 m*M* disodium EDTA, which were used to protect the monoamines and catechol isoquinolines from oxidation during the preparation procedure, and the sample was centrifuged at 22 000 *g* for 10 min at 4°C. The supernatant was transferred to a micro centrifugation tube and neutralized with 100 μl of 1 *M* potassium carbonate. The sample was centrifuged again at 22 000 *g* for 10 min at 4°C, and then filtered through a Millipore HV filter (pore size 0.45 μm).

2.4. HPLC analysis of the enantiomers of Sal and NMSal

The HPLC system consisted of a Shimadzu LC-9A pump (Kyoto, Japan), an electrochemical detector Coulochem-II (ESA, Bedford, MA, USA), an autosampler AS-8000 (Tosoh, Tokyo, Japan) equipped with a 25-μl sample loop and a Shimadzu C-R6A Chromatopac recorder. A chiral column (244 mm × 4.0 mm I.D.), LichroCART 250-4 ChiraDex (Merck, Darmstadt, Germany), was used for the separation of the enantiomers of Sal and NMSal. For the determination of dopamine and Sal enantiomers, coupled columns, a ChiraDex column connected with an Inertosil ODS-80A column (150 mm × 4.6 mm I.D., GL Sciences, Tokyo, Japan) in series was employed. The mobile phase consisted of a mixture of 0.1 *M* sodium phosphate buffer, pH 5.20, and 2.5% of methanol, and the flow-rate was 0.5 ml/min. The conditions of the Coulochem-II detector were as follows: a conditioning cell, Model 5021, was set at +300 mV, and the first electrode of an analytical cell, Model 5011, was at +50 mV and the second electrode at −300 mV. The output of the second electrode was monitored. Quantitation was performed by comparison of the peak area of the sample with that of the standard of Sals and NMSals. The separation factor (α) was calculated by the equation $\alpha = k'_2/k'_1$, where k' is the capacity ratio and calculated by $k' = (t_R - t_o)/t_o$: t_R is the retention time of the component and t_o

is that of the first peak in chromatogram as the hold-up time [28].

3. Results

The mixture of both Sal and NMSal enantiomers was analyzed with different kinds of mobile phase. The separation of the enantiomers was found to be dependent on the concentration and pH value of the buffer, as well as on the concentration and nature of the organic solvent. Increase in the polarity of the organic solvent (e.g. methanol) enhanced the separation. As buffer in the pH-stability range applicable for a ChiraDex column, Na_2HPO_4–NaH_2PO_4 was most effective for the separation of the enantiomers of Sal. Fig. 2 represents the effect of the buffer concentration, pH and methanol concentration on the separation of the enantiomers of Sal. The optimal mobile phase selected was a mixture of 0.1 *M* sodium phosphate buffer, pH 5.2, and methanol (97.5:2.5, v/v). The values of the separation factor (α) corresponding to the enantiomers of Sal and NMSal were 1.20 and 1.18, respectively. However, this column could not separate (*S*)-Sal from dopamine under the selected conditions, and the coupled columns were used for the samples containing dopamine. Fig. 3 shows that the racemic mixture of Sal and NMSal was well separated from each other and from their catecholamine precursor dopamine.

Fig. 4 illustrates the electrochemical behaviour of dopamine, Sal and NMSal, as determined by oxidation from 0 to 500 mV. In the voltage range from 300 mV to 400 mV, the responses of ECD for the five components reached a maximum. For analysis of biological samples, a redox mode was used to detect the compounds with the ECD conditions described in Section 2.

To determine the linearity of the method, various concentrations of diluted solution of racemic Sal and NMSal were quantitatively analyzed. Standard curves for the four compounds were obtained in the range of 0.125 to 5.0 pmol per injection (see Fig. 5). Good linear relationship was obtained over a range of concentration (0.125–5 pmol). The correlation coefficients for

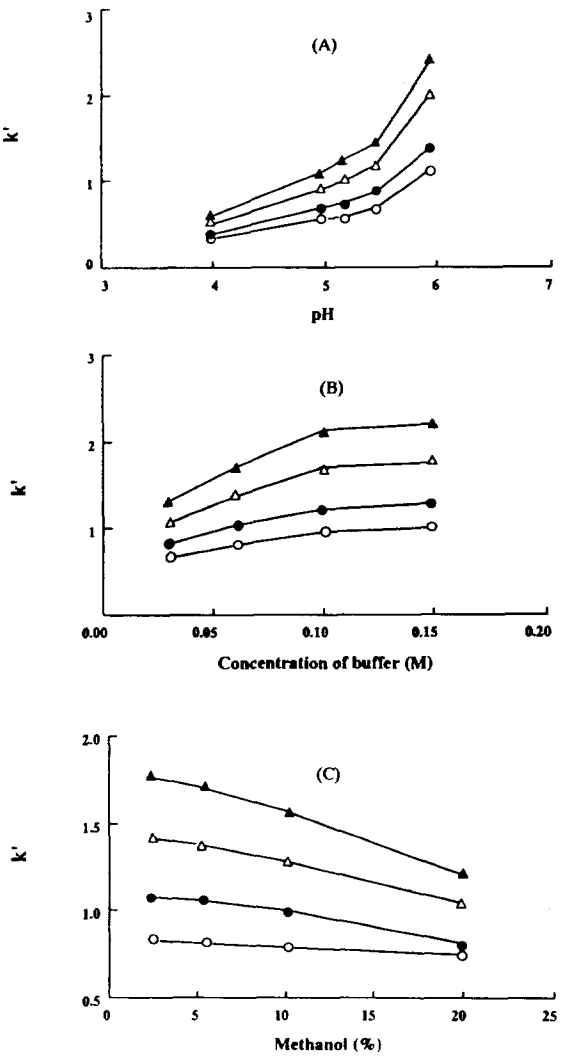

Fig. 2. Effect of pH value (A) and the concentration (B) of buffer, and the percentage of methanol (C) on the separation of the enantiomers of salsolinols and N-methylsalsolinols; ○ = (*S*)-Sal, ● = (*R*)-Sal, △ = (*S*)-NMSal, ▲ = (*R*)-NMSal; (A) 0.1 *M* phosphate buffer, 5% methanol; (B) pH 5.2, 5% methanol; (C) 0.1 *M* phosphate buffer, pH 5.2.

(*R*)- and (*S*)-Sal, and (*R*)- and (*S*)-NMSal were 0.97, 0.96, 0.98 and 0.97, respectively. The detection limits for the (*R*)- and (*S*)-enantiomers of Sal and NMSal were about 0.05 to 0.08 pmol per injection at a signal-to-noise ratio of 3, and shown in Table 1.

Fig. 3 shows the chromatograms of the condensation products formed in vitro by the Pictet–

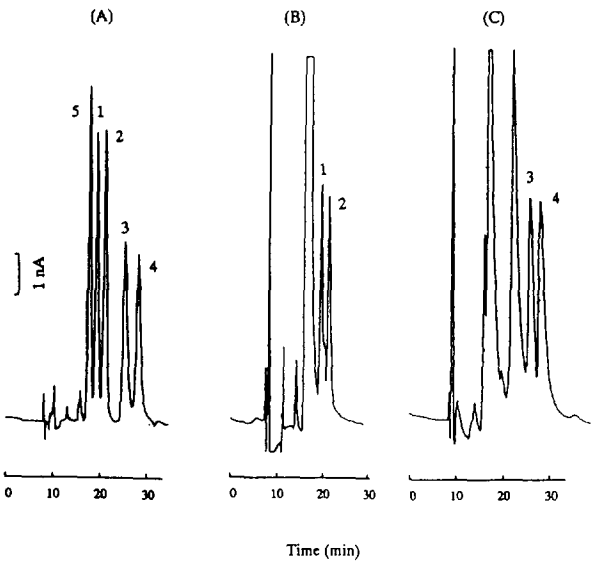

Fig. 3. Chromatograms of standard (A) and samples (B and C) from the Pictet–Spengler reaction. (A) Amount per injection for each component, 2.5 pmol. (B) The sample from the condensation of dopamine with acetaldehyde. (C) The sample from the in vitro synthesis of epinine and acetaldehyde. Peaks: 1 = (*S*)-Sal; 2 = (*R*)-Sal; 3 = (*S*)-NMSal; 4 = (*R*)-NMSal; 5 = dopamine.

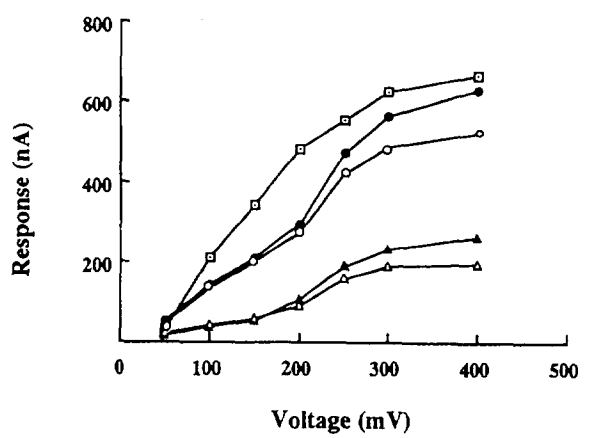

Fig. 4. The voltammograms of dopamine, salsolinols and N-methylsalsolinols. The voltage of the conditioning cell and the first electrode of a 5011 analytical cell were set at 0 mV, and the response of the second electrode of the analytical cell (expressed as nA) was plotted against the applied voltage. The response was measured in the oxidation mode. ○ = (*S*)-Sal, ● = (*R*)-Sal, △ = (*S*)-NMSal, ▲ = (*R*)-NMSal, □ = dopamine.

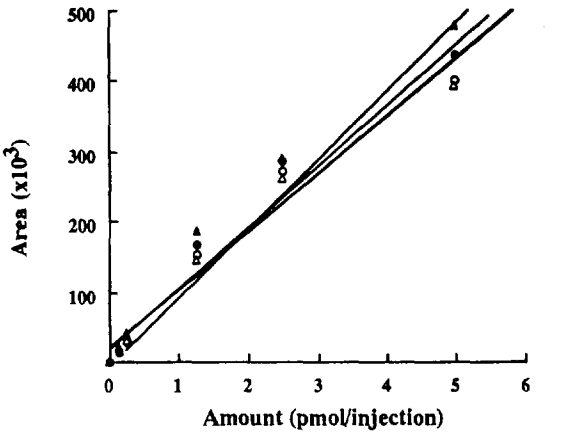

Fig. 5. The effects of the amounts of enantiomers of Sal and NMSal on the response of the second electrode of a 5011 analytical cell in a redox mode as described in Section 2. ○ = (*S*)-Sal, ● = (*R*)-Sal, △ = (*S*)-NMSal, ▲ = (*R*)-NMSal.

Spengler reaction of acetaldehyde with dopamine (B) or epinine (C). The (*R*)- and (*S*)-enantiomers of Sal and NMSal were separated and identified in the samples from the reaction mixture of acetaldehyde with dopamine or epinine. As shown in Fig. 6, the (*R*)/(*S*) ratio of enantiomers for both Sal and NMSal was found to be ca. 1 with different concentrations of catecholamines.

The method was used to measure the enantiomeric composition of Sal and NMSal in human brain samples. A typical chromatogram of a grey matter sample is shown in Fig. 7. Only the (*R*)-enantiomers of Sal and NMSal were detected in a sample from the human brain, and their amounts were 0.33 ± 0.03 and 0.53 ± 0.15

Table 1
Minimum amount detectable for (*R*)- and (*S*)-enantiomers of salsolinol and N-methylsalsolinol

Compound	Limit of detection ($S/N > 3$) (pmol/injection)
(*S*)-Sal	0.047
(*R*)-Sal	0.079
(*S*)-NMSal	0.060
(*R*)-NMSal	0.065

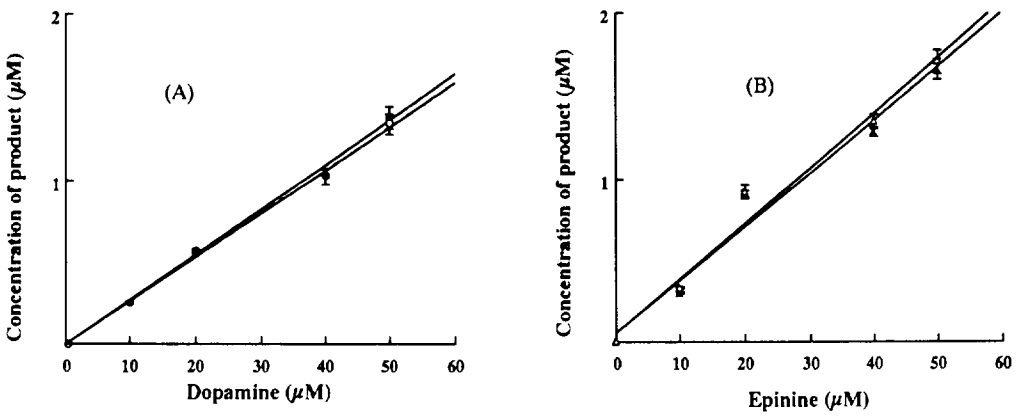

Fig. 6. Amounts and enantiomeric composition of salsolinols (A) and N-methylsalsolinols (B) from the Pictet–Spengler reaction under various concentrations of dopamine (A) or epinine (B) with 500 μM acetaldehyde. ○ = (*S*)-Sal, ● = (*R*)-Sal, △ = (*S*)-NMSal, ▲ = (*R*)-NMSal.

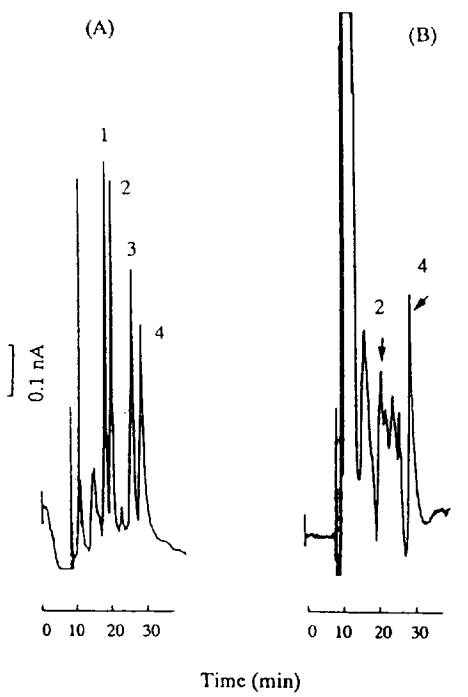

Fig. 7. Chromatograms of standard (A) and human brain sample (B). (A) Amount per injection for each standard component, 0.25 pmol. (B) The sample prepared from the grey matter of the human brain. Peaks: 1 = (*S*)-Sal; 2 = (*R*)-Sal; 3 = (*S*)-NMSal; 4 = (*R*)-NMSal.

nmol/g wet weight for (*R*)-Sal and (*R*)-NMSal, respectively.

4. Discussion

The separation of enantiomers by HPLC can be performed either by use of a chiral mobile or stationary phase, or by derivatization of the sample with a chiral reagent to form diastereoisomeric derivatives. The method described here for the separation of Sal and NMSal enantiomers made use of a chiral stationary phase, ChiraDex. The stationary phase is composed of spherical particles of silica gel modified with β-cyclodextrin. Cyclodextrins are built from chiral glucose units and have hydrophobic cavities which constitute chiral recognition sites. Inclusion complexes of β-cyclodextrin with (*R*)- and (*S*)-enantiomers might possess different association constants. Our results have demonstrated that the differences in the association constants of the complexes for both Sal and NMSal are large enough to allow discrimination of their enantiomers.

The method has several advantages over those previously described [14,24]. An HPLC–ECD method developed by Pianezzola et al. [24] was successful for to separate salsolinol enantiomers.

However, the derivatization to the diastereoisomers makes the method time-consuming and difficult to perform quantitatively. The present method eliminates the sample preparation steps, thus increasing the sensitivity of detection at a level of 0.1 pmol/injection. In addition, good accuracy and reproducibility were obtained by using this method due to the excellent resolution of these enantiomers. While this paper was submitted to this journal, Saellstroem Baum and Rommelspacher [29] reported the quantitation of (*R*)- and (*S*)-Sal in human plasma by a β-cyclodextrin-OH column (Macherey-Nagel, Duren, Germany). Even though these two methods use a similar type of HPLC column, cyclodextrin columns, the chromatographic conditions are different. Their method was not applied for the separation of enantiomers of NMSal and required the concentration of the sample prior to the HPLC analysis. In addition, they used only (*S*)-Sal as standard and identification of (*R*)-Sal was indirect, by comparison with the chromatogram of the racemic Sal mixture with (*S*)-Sal, while we used pure (*R*)- and (*S*)-enantiomers of Sal and NMSal for the identification. Our method reported here is simpler and has a wider application for the chiral separation of these isoquinolines.

In our experiment, Sal or NMSal was produced easily in vitro by the Pictet–Spengler condensation of acetaldehyde with dopamine or epinine, and the (*R*)/(*S*) ratio was demonstrated to be ca. 1 for both Sal and NMSal. The assay for human brain sample by this method revealed that only the (*R*)-enantiomers are present in human brain. On the other hand, in human plasma both (*R*)- and (*S*)-salsolinols were detected [29]. These results further suggest that under certain physiological conditions Sal in the human brain may be synthesized from the enzyme-catalyzing condensation of dopamine with pyruvic acid, followed by enzymatic decarboxylation and reduction to form (*R*)-Sal predominantly. The occurrence of (*R*)-Sal in the brain may be relevant to the previous data on Sal in human urine [24,25]. The detection of (*R*)-NMSal indicates that the enzymatic N-methylation of (*R*)-Sal [16] is the major biosynthesis pathway of (*R*)-NMSal in the human brain. The production of NMSal from epinine by the Pictet–Spengler reaction should not contribute to its occurrence in the brain.

This simple HPLC–ECD method provides a rapid, convenient, and sensitive means to determine the level and enantiomeric composition of Sal and NMSal in biological samples and foods. Recently, the metabolism of salsolinols and their possible involvement in the pathogenesis of neurodegenerative diseases, such as Parkinson's disease, have been reviewed [21]. N-methylation of Sal is the first reaction required for the selective uptake into dopamine neurons, since only (*R*)-NMSal was found to be transported into the cell by the dopamine transporter [22]. The method will be applicable to the study of the metabolism and the biological and pharmacological effects of these compounds.

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References

[1] M. Sandler, S. Bonham Carter, K.R. Hunter and G.M. Stern, Nature, 241 (1973) 439.
[2] M.A. Collins, W.P. Nijm, G.F. Borge, G. Teas and C. Golfard, Science, 206 (1979) 1184.
[3] B. Sjoequist, S. Borg and H. Kvande, Subst. Alc. Act. Misuse, 2 (1981) 73.
[4] G. Dordain, P. Dostert, M. Strolin Benedetti and V. Rovei, in K.F. Tipton, P. Dostert, M. Strolin Benedetti (Editors), Monoamine Oxidase and Disease. Prospects for Therapy with Reversible Inhibitors, Academic Press, London, 1984, p. 417.
[5] B. Sjoequist, A. Eriksson and B. Winblad, Prog. Clin. Biol. Res., 90 (1982) 57.
[6] T. Niwa, N. Takeda, H. Yoshizumi, A. Tatematsu, M. Yoshida, P. Dostert, M. Naoi and T. Nagatsu, Biochem. Biophys. Res. Commun., 177 (1991) 603.
[7] P. Dostert, M. Strolin Benedetti and G. Dordain, J. Neural Transm., 74 (1988) 61.

[8] M. Naoi, W. Maruyama, P. Dostert, D. Nakahara, T. Takahashi and T. Nagatsu, in I. Hanin, M. Yoshida and A. Fisher (Editors), Alzheimer's and Parkinson's Diseases: Recent Developments, Plenum, New York, NY, 1995, p. 553.
[9] G.A. Smythe and M.W. Duncan, Prog. Clin. Biol. Res., 183 (1985) 77.
[10] M. Strolin Benedetti, P. Dostert and M. Dedieu, in 15th C.I.N.P. Congress, Puerto Rico, December 1986, p. 56.
[11] M. Strolin Benedetti, P. Dostert and P. Carminati, J. Neural Transm. [GenSect], 78 (1989) 43.
[12] T. Origitano, J. Hannigan and M.A. Collins, Brain Res., 224 (1981) 446.
[13] P. Dostert, M. Strolin Benedetti, V. Bellotti, C. Allievi and G. Dordain, J. Neural Transm. [GenSect], 81 (1990) 215.
[14] M. Strolin Benedetti, V. Bellotti, E. Poanezola, E. Moro, P. Carminati and P. Dostert, J. Neural Transm., 77 (1989) 47.
[15] M. Kajita, T. Niwa, N. Takeda, H. Yoshizumi, A. Tatematsu, K. Watanabe and T. Nagatsu, J. Chromatogr., 613 (1993) 1.
[16] W. Maruyama, D. Nakahara, M. Ota, T. Takahashi, A. Takahashi, T. Nagatsu and M. Naoi, J. Neurochem., 59 (1992) 395.
[17] M. Kajita, T. Niwa, W. Maruyama, D. Nakahara, N. Takeda, H. Yoshizumi, A. Tatematsu, K. Watanabe, M. Naoi and T. Nagatsu, J. Chromatogr. B, 654 (1994) 263.
[18] G. Cohen, R.E. Heikkila, D. Dembiec, D. Sang, S. Teitel and A. Brossi, Eur. J. Pharmacol., 29 (1974) 292.
[19] M. Minami, W. Maruyama, P. Dostert, T. Nagatsu and M. Naoi, J. Neural Transm. [GenSect], 92 (1993) 125.
[20] M. Minami, T. Takahashi, W. Maruyama, A. Takahashi, P. Dostert, T. Nagatsu and M. Naoi, J. Neurochem., 58 (1992) 2097.
[21] M. Naoi, W. Maruyama and P. Dostert, Prog. Brain Res., (1995), in press.
[22] T. Takahashi, Y. Deng, W. Maruyama, P. Dostert, M. Kawai and M. Naoi, J. Neural Transm. [GenSect], 98 (1994) 107.
[23] P. Dostert, M. Strolin Benedetti and M. Dedieu, Pharmacol. Toxicol., 60 (Suppl. 1) (1987) 12.
[24] E. Pianezzola, V. Bellotti, E. Fontana, E. Moro, J. Gal and D.M. Desai, J. Chromatogr., 495 (1989) 205.
[25] P. Dostert, M. Strolin Benedetti, G. Dordain and D. Vernay, J. Neural Transm., [P-D Sect], 1 (1989) 269.
[26] P. Dostert, M. Strolin Benedetti, G. Dordain and D. Vernay, J. Neural Transm. [GenSect], 85 (1991) 51.
[27] S. Teitel, J. O'Brein and A. Brossi, J. Med. Chem., 15 (1972) 845.
[28] R.M. Smith, Gas and Liquid Chromatography in Analytical Chemistry, Wiley, Chichester, 1988, p. 20.
[29] S. Saellstroem Baum and H. Rommelspacher, J. Chromatogr. B, 660 (1994) 235.