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Assay for the (*R*)- and (*S*)-enantiomers of salsolinols in biological samples and foods with ion-pair high-performance liquid chromatography using β-cyclodextrin as a chiral mobile phase additive

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Abstract

A chromatographic procedure was devised for the quantitative determination of the enantiomers of salsolinol and N-methylsalsolinol, which are biologically important alkaloids. The enantiomers of salsolinol and N-methylsalsolinol were completely separated using β-cyclodextrin in a reversed-phase ion-pair system. The HPLC method was sensitive enough to detect the isoquinolines at a concentration less than 0.1 pmol per injection. The presence of (*R*)- and (*S*)-salsolinol was confirmed in fermented foods and beverages, while N-methylsalsolinol was not detected. On the other hand, the (*R*)-enantiomers of both salsolinol and N-methylsalsolinol were found to predominate in the human brain.

Keywords: Enantiomer separation; Salsolinol; N-Methylsalsolinol

1. Introduction

6,7 - Dihydroxy - 1 - methyl - 1,2,3,4 - tetrahydroiso - quinoline (salsolinol, Sal) was found to occur in human brain [1] and to be a precursor of an endogenous neurotoxin, 6,7-dihydroxy-1,2-dimethyl-1,2,3,4-tetrahydroisoquinoline (N-methylsalsolinol, NMSal) [2]. NMSal was detected in human brain by GC–MS [3], and N-methylation of Sal into NMSal was demonstrated by in vivo microdialysis in the rat brain [4]. Sal and NMSal have an asymmetric center at C_1 and exist as (*R*)- and (*S*)-enantiomers. Sal has been considered to be formed from dopamine, either by a non-enzymatic Pictet–Spengler reaction to yield racemic (*RS*)-Sal, or by an enzymatic reaction to produce the (*R*)-enantiomer. Sal was also found in some foods and beverages, such as banana and port wine, suggesting that the intake of Sal might affect Sal levels in tissues [5]. Recently, different biological characteristics of (*R*)- and (*S*)-enantiomers of Sal and NMSal were reported. (*R*)-Sal was found to stronger inhibit type A of monoamine oxidase

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[monoamine: oxygen oxidoreductase (deaminating), EC 1.4.3.4] than its enantiomeric counterpart [6]. The chirality of Sal and NMSal was found to affect the affinity of tyrosine hydroxylase [tyrosine, tetrahydrobiopterin: oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2] to a substrate, L-tyrosine [7]. After injection of these alkaloids in rat brain, only (*R*)-NMSal caused behavioral changes similar to those of patients with Parkinson's disease, but (*R*)- and (*S*)-Sal, and (*S*)-NMSal did not [8]. Using human dopaminergic neuroblastoma SH-SY5Y cells, only (*R*)-NMSal was taken up in the cells selectively by the dopamine uptake system [9]. These results suggest that the stereochemical structure of Sal and NMSal may be involved in their biological functions.

A few papers reported the determination of the enantiomeric ratio of Sal. A HPLC method was achieved after methylation with diazomethane and then derivatization to diastereomers with N-trifluoroacetyl-L-prolyl chloride [10]. For a better separation, a chiral derivatizing agent, (*S*)-1-(1-naphthyl)ethyl isothiocyanate, was used, and the enantiomeric composition of Sal in human urine samples was determined by this assay [11]. In all the above-mentioned methods, the enantiomers of Sal must be first derivatized to a pair of diastereoisomers. Recently, we devised a HPLC–electrochemical detection (ED) method for the quantification of enantiomers of Sal and NMSal without derivatization by use of a cyclodextrin-bonded column [12]. This method is sensitive enough to determine Sal and NMSal in human brain samples, but the column was fragile for the application of crude biological samples.

Recently, cyclodextrins have been increasingly applied as mobile phase additives in HPLC. There are several advantages to the use of chiral selectors in a mobile phase. Less expensive conventional packed columns (such as reversed-phase) can be used. The type and concentration of the cyclodextrins used as additives can be easily changed. In this work, the direct enantioseparation of Sal and NMSal could be achieved using a reversed-phase column with aqueous mobile phase containing β-cyclodextrin and a counter ion; then this novel method was applied to determine the enantiomeric composition of Sal and NMSal in human brain and some food samples.

2. Experimental

2.1. Chemicals and reagents

Both (*R*)- and (*S*)-Sal, (*R*)- and (*S*)-NMSal were synthesized according to Teital et al. [13]. 6,7-Dihydroxy-2-methyl-1,2,3,4-tetrahydroisoquinoline (N-methylnorsalsolinol, NMNorsal) was purchased from Janssen (Beerse, Belgium). Dopamine was purchased from Sigma (St. Louis, MO, USA), and β-cyclodextrin (β-CD) from Nacalai tesque (Kyoto, Japan). An ion-pair reagent, sodium 1-heptanesulfonate (SHS), was obtained from Sigma. A Varian Bond Elut PSA (primary/secondary amines) and PBA (phenylboronic acid) cartridges (Harbor City, CA, USA) were used for solid-phase extraction. All other chemicals were of analytical grade and organic solvents were of HPLC grade from Nacalai tesque.

2.2. Preparation of human brain and some food samples

The extraction of catechol isoquinolines was carried out as described by Sällström Baum and Rommelspacher with a slight modification [14]. Gray matter of human brain (1.0 g wet weight) was suspended in five volume/wet weight (5 ml) of 0.1 *M* perchloric acid containing 0.1 m*M* sodium metabisulfite and disodium EDTA, and sonicated in a Branson sonicator (Danbury, CT, USA) for 1 min with 50% duty cycle. The homogenate was centrifuged at 20 000 *g* for 30 min at 5°C, and the supernatant was mixed with equivolume of a solution A prepared by dissolving 13.21 g of $(NH_4)_2SO_4$ in 100 ml of 0.11 *M* NaOH, whose pH was adjusted to 8.5. For the selective extraction of Sal and NMSal and dopamine, a PSA (N-propylethylenediamine, 100 mg/ml) and a PBA (phenylboronic acid, 100 mg/ml) cartridge were connected together in series. The cartridges were conditioned as follows: firstly only the PSA cartridge was washed with 1 ml of methanol and 1 ml of 0.1 *M* HCl, and the PSA cartridge was connected to the PBA cartridge, then they were washed successively with 1 ml of methanol, 1 ml of a solution diluted 50-fold from solution A with distilled water, and 5 ml of 5 m*M* sodium

phosphate buffer (pH 8.5). The sample solution was loaded on the combined PSA–PBA cartridges. After washing with 5 ml of 5 m*M* phosphate buffer (pH 8.5) and subsequently with 1 ml methanol, the PSA cartridge was removed and the catecholamines were eluted with 3 ml of 0.1 *M* HCl from the PBA cartridge. The eluate was evaporated to dryness under vacuum and dissolved in 500 μl of distilled water.

Catecholisoquinolines were extracted from two brands of wine samples, German and French wine. A 2-ml volume of 1 *M* HCl was added to 18 ml of wine, and the mixture was centrifuged at 20 000 *g* for 45 min. The supernatant was brought to pH 8.5 with 3.5–4.5 ml of 1 *M* NaOH, and centrifuged again at 20 000 *g* for 45 min. Then the sample solution was submitted to selective extraction on PSA+PBA cartridges, as described above.

Dried banana sample was homogenized in ten volumes/weight of 0.1 *M* HCl and centrifuged at 22 000 *g* for 10 min at 4°C. The supernatant was diluted 100× with distilled water, then filtered through a Millipore HV filter.

2.3. HPLC apparatus and chromatographic conditions

The HPLC system was composed of a Shimadzu LC-9A pump (Kyoto, Japan), an electrochemical detector Coulochem-II (ESA, Chelmsford, MA, USA), an autosampler AS-8010 (Tosoh, Tokyo, Japan) and a Shimadzu C-R6A chromatopac recorder (Kyoto, Japan). The separation was performed using a reversed-phase Inertosil ODS-3 column (250×4.6 mm I.D., GL Sciences, Tokyo, Japan). The mobile phase consisted of 25 m*M* sodium phosphate buffer, pH 3.0, containing 12 m*M* β-cyclodextrin, 1 m*M* SHS and 3% acetonitrile, 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. The retention time of the first peak in the chromatogram was used as the hold-up time (t_0). Data represents the average of three or more analyses.

3. Results

Fig. 1 represents the structures of Sal and NMSal enantiomers, and their precursor dopamine.

Fig. 2A shows a typical chromatographic pattern of the standard mixture of dopamine, and Sal and NMSal enantiomers, obtained using a chiral mobile phase consisting of 25 m*M* phosphate buffer (pH 3.0), 12 m*M* β-cyclodextrin, 1 m*M* SHS and 3% acetonitrile. A suitable structural analog, N-methylnorsalsolinol (NMNorsal), was selected as an internal standard. The HPLC procedure provides excellent separation for all six components.

The linearity and reproducibility of HPLC analyses of the standards were examined without solid-phase extraction. Fig. 3 shows the dependence of the relative responses of the analytes on the analyte concentration. Good linear relationship between concentrations and relative responses was obtained over the measured ranges from 2.0 to 40 pmol per injection and from 0.1 to 2.0 pmol per injection with EC sensitivity of 0.1 or 0.01 μA full scale, respectively. The correlation coefficients for dopamine, (*R*)- and (*S*)-Sal, and (*R*)- and (*S*)-NMSal were from 0.96 to 0.99. The detection limits for dopamine and (*R*)- and (*S*)-enantiomers of Sal and NMSal were 0.082, 0.047, 0.065, 0.061 and 0.073 pmol per

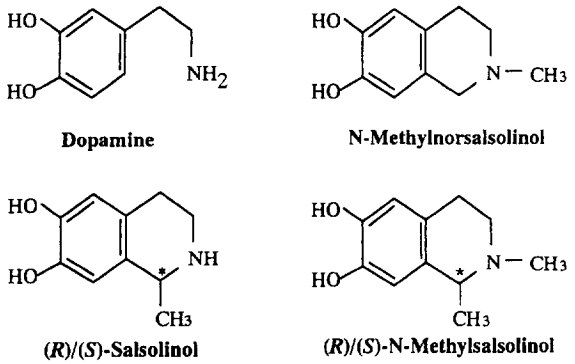

Fig. 1. Structures of dopamine, NMNorsal and (*R*)- and (*S*)-enantiomers of Sal and NMSal.

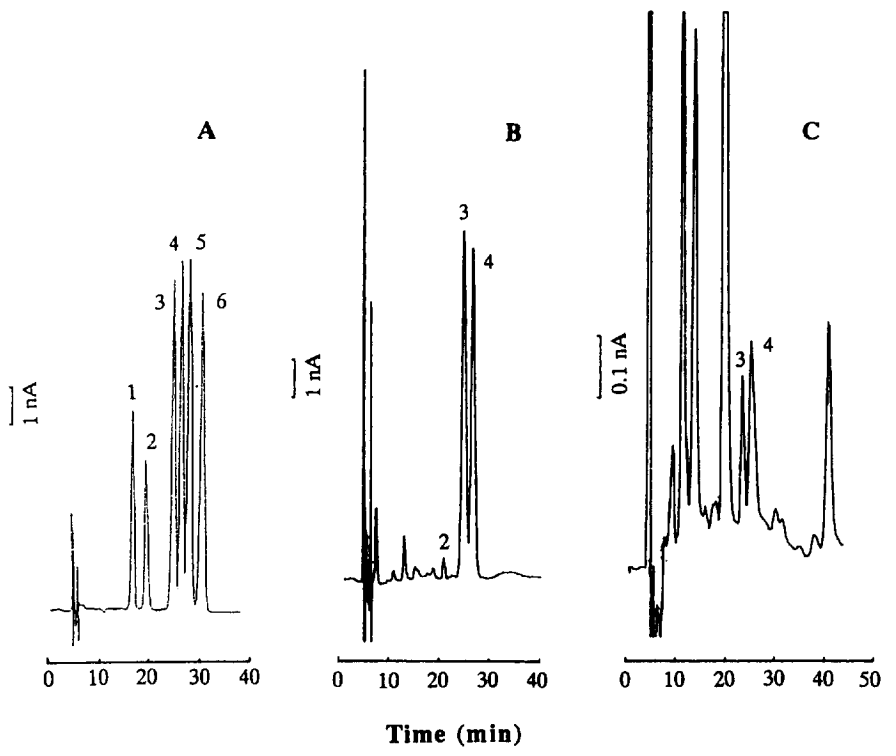

Fig. 2. HPLC patterns of samples prepared from dried banana and wine sample. (A) Amount per injection for each component, 20 pmol; (B) sample prepared from dried banana; (C) sample prepared from red German wine. The mobile phase: 25 m*M* phosphate buffer, pH 3.0, containing 12 m*M* β-cyclodextrin, 1 m*M* SHS and 3% acetonitrile. Peaks: 1=NMNorsal; 2=dopamine; 3=(*R*)-Sal; 4=(*S*)-Sal; 5=(*R*)-NMSal; 6=(*S*)-NMSal.

injection at a signal-to-noise ratio of three, respectively.

A standard mixture containing 10 n*M* dopamine, Sal enantiomers, NMSal enantiomers and NMNorsal was submitted to the extraction procedure. The extraction recoveries (%) were 77.8±9.2 for dopamine, 86.2±8.4 for Sal enantiomers, 75.7±8.5 for NMSal enantiomers and 52.3±9.0 for the internal standard NMNorsal (mean±S.D., $n=9$). The recoveries were determined over the concentration range of isoquinolines from 5 to 50 n*M*, and were almost constant. The recovery of NMNorsal was relatively low, the exact reason for this remains unclear. To investigate the method validation, inter-day precision and accuracy of the assay of five components of interest were determined. Brain sample was spiked with dopamine, (*R*)- and (*S*)-Sal, (*R*)- and (*S*)-NMSal, and NMNorsal with the amounts of 0.1 nmol in 5 ml of the supernatant for each component, and subjected to extraction and concentration. The intra-day and inter-day precision of this assay is shown in Table 1 and the C.V.s of the inter-day precision (within two days) were found to be 7.0, 12.9, 14.0, 13.1 and 13.8% for dopamine, (*R*)-Sal, (*S*)-Sal, (*R*)-NMSal and (*S*)-NMSal, respectively. Table 2 summarizes the recoveries of the method for dopamine, (*R*)- and (*S*)-enantiomers of Sal and NMSal.

Using this assay, we have measured the enantiomeric composition of Sal and NMSal in human brain. A typical HPLC pattern of a human brain sample prepared from gray matter is shown in Fig. 4. Only the (*R*)-enantiomers of Sal and NMSal were detected in a sample prepared from human brain (see Table 3), and their amounts were 1139±136, 71.4±28.1, and 96.2±36.4 pmol/g wet weight (mean±S.D., $n=11$) for dopamine, (*R*)-Sal and (*R*)-NMSal, respectively.

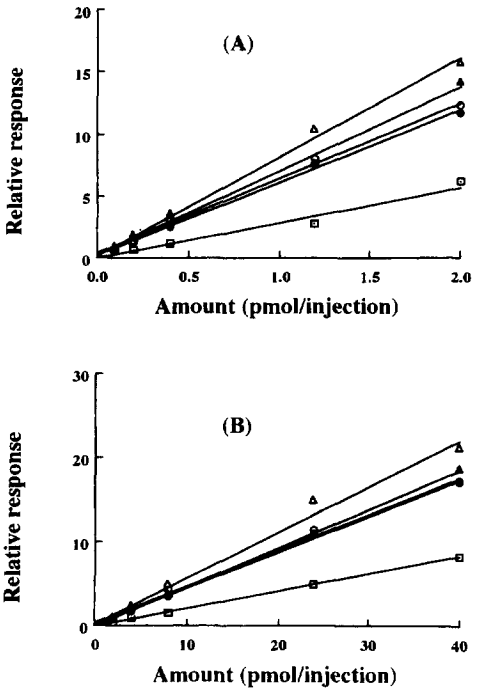

Fig. 3. Effects of the amounts of analytes on the relative responses [the peak area of the analyte was expressed as the ratio to that of NMNorsal (4 pmol/injection)]. (A) The amounts of analytes in the range from 0.1 to 2.0 pmol per injection with sensitivity of 0.01 μA full scale. (B) The amounts of analytes in the range from 2.0 to 40 pmol per injection with sensitivity of 0.1 μA full scale. (□) Dopamine, (○) (*R*)-Sal, (●) (*S*)-Sal, (△) (*R*)-NMSal, (▲) (*S*)-NMSal.

Two brands of wine purchased locally, German and French, were analyzed, and both (*R*)- and (*S*)-enantiomers of Sal were clearly present in these samples. In addition, dried banana was found to contain high concentrations of (*R*)- and (*S*)-enantiomer of Sal in equimolar amount. In all measured beverage and food samples, NMSal could not be detected by this assay. Fig. 2B and Fig. 2C show typical chromatograms of samples from dried banana and wine. The amounts of dopamine, Sal and NMSal in foods and beverages and human brain are summarized in Table 3.

4. Discussion

The use of cyclodextrins as chiral mobile phase additives in a reversed-phase HPLC system has been reported by several research groups. Generally, there are several factors required for chiral separation. The formation constant of the inclusion complex of cyclodextrin with a guest analyte must be sufficiently large and markedly different between the enantiomers, and retention must also be high enough. The formation of an inclusion complex is mainly determined by the relative size of the host cyclodextrin cavity. Our previous results on the separation of the enantiomers of Sal and NMSal by use of a β-cyclodextrin-bonded column, suggest that the cavity of β-cyclodextrin is compatible with Sal and NMSal molecules [12]. The concentration of β-cyclodextrin in the mobile phase is of importance for the enantioselectivity. The optimal concentration was found to be 12 m*M* for the separation of all components of interest in this assay, since a higher concentration of β-cyclodextrin made the peaks of (*S*)-Sal and (*R*)-NMSal overlap.

Relatively hydrophilic amines like Sal and NMSal are not sufficiently retained on a reversed-phase column, although the retention is essential for the enantioseparation. Derivatized cyclodextrins were usually used to enhance retention of analytes on the

Table 1
Intra-day and inter-day precision of this assay for dopamine, Sal and NMSal enantiomers

Compound	Intra-day		Inter-day (within two days)	
	Amount[a] (nmol) (mean±S.D., $n=3$)	C.V. (%)	Amount[a] (nmol) (mean±S.D., $n=6$)	C.V. (%)
Dopamine	1.228±0.092	7.5	1.249±0.087	7.0
(*R*)-Sal	0.170±0.019	11.2	0.162±0.021	12.9
(*S*)-Sal	0.083±0.011	13.2	0.086±0.012	14.0
(*R*)-NMSal	0.191±0.027	14.1	0.183±0.024	13.1
(*S*)-NMSal	0.112±0.011	9.8	0.109±0.015	13.8

[a] The supernatant of human brain sample (5 ml) was spiked with 0.1 nmol of dopamine, Sal enantiomers and NMSal enantiomers.

Table 2
Accuracy of the assay for dopamine, Sal and NMSal enantiomers

Compounds	Amount[a] spiked (nmol)	Endogenous amount (nmol)	Amount found (mean±S.D., $n=6$) (nmol)	Recovery[b] (%)
Dopamine	0.1	1.139	1.249±0.087	110
(*R*)-Sal	0.1	0.0714	0.162±0.021	90.6
(*S*)-Sal	0.1	ND[c]	0.086±0.012	86.0
(*R*)-NMSal	0.1	0.0962	0.183±0.024	86.8
(*S*)-NMSal	0.1	ND	0.109±0.015	109

[a] The supernatant of human brain sample (5 ml) was spiked with 0.1 nmol of dopamine, Sal enantiomers and NMSal enantiomers.
[b] Recoveries were calculated by which the values of amount found were subtracted by the endogenous amounts in brain sample, and then compared with the spiked amounts.
[c] ND=not detected.

C_{18} stationary phase. In this work, new chromatographic conditions were devised for the resolution of enantiomers of Sal and NMSal, where unmodified β-cyclodextrin was used in a ion-pair mobile phase containing SHS as a counter ion with a reversed-phase column. This procedure could achieve the enantioseparation of Sal and NMSal conveniently and effectively. Small amounts of SHS significantly enhanced the absorption of Sal and NMSal on a reversed-phase column by means of the increase in the hydrophobicity. It was suggested that the use of the counter ion not only increases the retention but also the stability of the β-cyclodextrin complexes with Sal enantiomers and NMSal enantiomers.

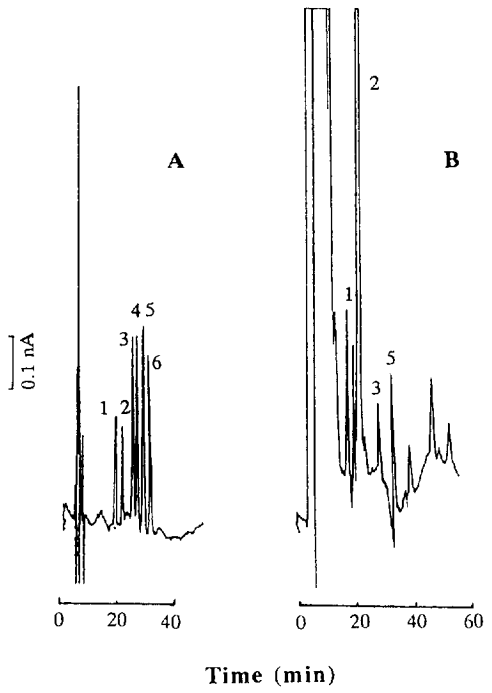

Fig. 4. HPLC patterns of samples prepared from human brain sample. (A) Standards, amount per injection for each standard component, 0.5 pmol. (B) Sample prepared from human brain gray matter. The mobile phase: 25 m*M* phosphate buffer, pH 3.0, containing 12 m*M* β-cyclodextrin, 1 m*M* SHS and 3% acetonitrile. Peaks: 1=NMNorsal; 2=dopamine; 3=(*R*)-Sal; 4=(*S*)-Sal; 5=(*R*)-NMSal; 6=(*S*)-NMSal.

Formation of an inclusion complex with cyclodextrin requires aqueous media, but an aqueous–organic solvent mixture may be more efficient for separation. The selection of the organic solvent is of importance for the solubility of cyclodextrin, and the retention behavior of analytes, as well as for the stability of the inclusion complexes. In the present study, the best conditions for identification of these isoquinolines were found to use 3% acetonitrile, although 2-methyl-2-propanol was recommended for the β-cyclodextrin-containing mobile phase in several papers [15]. Under the selected conditions, the capacity ratios of dopamine, Sal enantiomers and NMSal enantiomers were between 2 and 10, the range recommended for optical resolution.

The use of a chemically bonded ion-exchange cartridge improved reproducibility and yield during the extraction process. A PBA cartridge was used to extract Sal from biological samples such as urine and plasma in several reports [10,11]. However, with only a PBA cartridge, the recovery of NMSal enantiomers was found to be very low. In this assay, two cartridges, PSA+PBA, were used in series, and the recoveries of both Sal and NMSal are quantitative. The reproducibility and recovery for analysis of

Table 3
Amounts of dopamine, Sal and NMSal enantiomers in the human brain and in some beverages and foods ($n=11$)

Compound	Gray matter[a] (pmol/g wet weight)	Dried banana (nmol/g wet weight)	German wine (pmol/ml)	French wine (pmol/ml)
Dopamine	1139±136	6.143±0.566	NA[b]	NA
(*R*)-Sal	71.4±28.1	364.6±6.1	34.5±4.5	18.5±7.1
(*S*)-Sal	ND[c]	343.9±15.2	43.8±12.6	27.5±9.8
(*R*)-NMSal	96.2±36.4	ND	ND	ND
(*S*)-NMSal	ND	ND	ND	ND

[a] Gray matter of human brain was obtained from patients without neurological history.
[b] NA=not assayed.
[c] ND=not detected.

these catecholamines spiked into brain sample, were found to satisfy recommended guidelines (15%) for assay precision and accuracy [16].

The presence of (*R*)- and (*S*)-enantiomers of Sal was confirmed in fermented food and beverage samples, such as banana and wine in our experiment. However, NMSal, a N-methylated derivative of Sal, was not detected in all these food and beverages. Our data for (*R*)- and (*S*)-Sal and for dopamine are in the same order of magnitude as those reported by Smythe and Duncan, where Sal was determined as the sum of (*R*)- and (*S*)-enantiomers [17]. The *R*/*S* ratio was found to be very near to 1 for Sal enantiomers in dried banana, which suggests the formation of Sal from dopamine and acetaldehyde by a nonenzymatic Pictet–Spengler reaction during the ripening process. Strolin Benedetti et al. reported the influence of food intake on the enantiomeric Sal composition in urine [4,10,18]. It was found that the (*R*)-enantiomer predominates in the urine of healthy volunteers and that after intake of dried banana (100 g) – which contain a high concentration of Sal – the (*S*)-enantiomer was detected in urine from subjects for which (*S*)-Sal had not been found before the intake. It is known, however, that Sal cannot be transported into the brain through the blood–brain barrier, so that the Sal detected in the brain must have been synthesized in situ [19]. Our present study revealed that (*R*)-enantiomers of Sal and NMSal are predominantly present in human brain. These results further suggest that under certain physiological conditions the enantiospecific biosynthesis of (*R*)-Sal may occur by enzymatic condensation. Recently, stepwise enzymatic reactions were found to be involved in the biosynthesis of (*R*)-Sal and further (*R*)-NMSal [20]. A (*R*)-salsolinol synthase, which catalyzes the biosynthesis of (*R*)-Sal from dopamine and acetaldehyde, was isolated from the human brain for the first time [21]. The other enzyme is an N-methyltransferase which produces NMSal in the brain [4]. The recent success of our animal model of Parkinson disease suggests that these two enzymes and their catalytic products may be involved in the pathogenesis of Parkinson's disease. This might be clarified by the analysis of these catechol isoquinolines in human brains using this novel HPLC method.

Acknowledgments

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