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Quantification of salsolinol enantiomers by stable isotope dilution liquid chromatography with tandem mass spectrometric detection

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Abstract

Salsolinol, 1-methyl-6,7-dihydroxy-2,3,4,5-tetrahydroisoquinoline (SAL), is a precursor of a Parkinsonian neurotoxin, N-methylsalsolinol (N-methyl-SAL). Previous studies have shown that individual enantiomers of N-methyl-SAL possess distinct neurotoxicological properties. In this work, a chiral high-performance liquid chromatography (HPLC) method with electrospray ionization tandem mass spectrometric (ESI-MS/MS) detection was developed for the quantification of (*R/S*)-SAL enantiomers. Enantioseparation was achieved on a β -cyclodextrin-bonded silica gel column, and the resolved enantiomers were detected by ESI-MS/MS operated in positive ion mode. The ESI collision-induced dissociation (CID) mass spectrum of SAL was studied together with that of its deuterium-labeled analog (i.e. salsolinol- $\alpha,\alpha,\alpha,1$ -d₄, SAL-d₄) so that the fragmentation pathways could be elucidated. Further, using SAL-d₄ as internal standard in HPLC/MS/MS analysis of SAL improved significantly assay accuracy and reliability. Determination of (*R/S*)-SAL enantiomers present in food samples such as dried banana chips was demonstrated.

Certain 1,2,3,4-tetrahydroisoquinoline derivatives (TIQs) exhibit neurotoxicological properties similar to those of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is a well-known neurotoxin causing Parkinsonism in humans, monkeys, and various animals.^{1,2} Therefore, the study of the neurotoxicity of TIQs has been intensive.^{3–5} N-Methylsalsolinol (N-methyl-SAL) formed via N-methylation of salsolinol (6,7-dihydroxy-1-methyl-1,2,3,4-tetrahydroisoquinoline, SAL) was shown to cause Parkinsonism in rats (see Fig. 1 for chemical structures). More interestingly, enantiomeric N-methyl-(*R*)-SAL was found to be 1000 times more potent in inducing Parkinsonism than N-methyl-(*S*)-SAL.^{4,6} The level of N-methyl-(*R*)-SAL has been found to be significantly higher in the cerebrospinal fluid of newly diagnosed Parkinsonian patients due to an increase of (*R*)-SAL N-methyltransferase activity.^{7–10} SAL has been detected in human brain, plasma, and urine.^{9–12} Although SAL can be formed *in vivo* from the condensation of dopamine with acetaldehyde, it can also be taken in from food consumption. SAL exists in various foods such as cheese, banana, broiled beef, wine, and milk.^{13–15}

Since individual enantiomers of N-methyl-(*R/S*)-SAL exert distinct neurotoxic effects, enantiomeric quantification of its precursor, (*R/S*)-SAL, in a variety of biological specimens has been receiving great research interest. Separation of (*R/S*)-SAL enantiomers has been achieved by using methods based on chiral high-performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS). A chiral GC/MS procedure deploying two-step pre-column derivatization to convert (*R/S*)-SAL enantiomers into volatile diastereoisomers was developed for the determination of dopamine, SAL, and norSAL stereoisomers in human brain.¹⁶ We developed a GC/MS method for separating (*R/S*)-SAL

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enantiomers on a cyclodextrin chiral GC column after a one-step pre-column derivatization to convert (*R/S*)-SAL into their volatile derivatives with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide.¹⁷ The method was applied to quantify free SAL enantiomers in the central nervous system of *Aplysia*. Since SAL is hydrophilic and easily oxidized in basic solutions, the cumbersome sample pretreatment and pre-column derivatization procedures required in GC/MS analysis can be problematic causing significant loss of SAL from the samples. A method based on ion-pairing HPLC with electrochemical detection was developed for enantiomeric determination of SAL.¹⁸ In this method, β -cyclodextrin was added into the mobile phase as chiral selector. Chiral HPLC methods with the use of cyclodextrin-bonded chiral columns were also developed.^{19,20} A comparative study on various HPLC chiral stationary phases for separating SAL enantiomers was reported.²¹ Most of these chiral HPLC methods employed electrochemical detection. Although these methods were very sensitive, they lacked the capability of peak identification and thus detection selectivity. In addition, these chiral HPLC separations employed mobile phases containing non-volatile components such as phosphate buffer that contaminate an MS detector. HPLC methods with mass spectrometric detection have been developed for the determination of TIQs without enantiomeric selectivity.^{22,23} A chiral HPLC/electrospray ionization tandem mass spectrometry (ESI-MS/MS) method was developed for simultaneous determination of SAL enantiomers and dopamine in human plasma and cerebrospinal fluid.²⁴ However, the method involved pre-column derivatization of the analytes with pentafluorobenzyl bromide (PFBBR) in order to achieve the enantioseparation. The enantiomeric tri-PFB-SAL derivatives were separated on a polysaccharide chiral column. Very recently, HPLC/MS/MS determination of catecholamines and SAL enantiomers present in rat brain tissues was reported.²⁵ The chiral separation was achieved on a β -CD column. No pre-column derivatization was involved in the method.

The aim of this work was to develop a chiral HPLC/MS/MS method for quantifying SAL enantiomers requiring minimum sample pretreatment and no pre-column derivatization. Our previous study showed that TIQs including SAL could be sensitively and selectively detected by ESI-MS/MS.²² The biggest challenge was to achieve an acceptable resolution of intact SAL enantiomers with a mobile phase that was compatible with MS detection. A range of chiral stationary phases (CSPs) including polysaccharide and cyclodextrin-bonded CSPs were evaluated for this purpose. An effective mobile phase was developed for the enantioseparation on cyclodextrin-bonded CSP. Mass spectrometric properties of SAL in an ion trap spectrometer were studied to ensure a high sensitivity and specificity of MS/MS detection. A stable isotope labeled analog of SAL (salsolinol- $\alpha,\alpha,\alpha,1$ -d₄, SAL-d₄) was synthesized and used for the elucidation of CID fragmentation mechanism and for isotope dilution HPLC/MS/MS of SAL. Quantification of (*R/S*)-SAL enantiomers in foodstuff samples by using the present method with stable isotope dilution was demonstrated.

EXPERIMENTAL

Chemicals

Racemic SAL (C₁₀H₁₃NO₂·HCl), dopamine, acetaldehyde-d₄, acetic acid, NH₄OH solution, methanol, isopropanol, and acetonitrile (HPLC grade) were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Deuterium-labeled racemic (*R/S*)-SAL-d₄·HCl was prepared in our lab following a procedure described by Shiraha *et al.*²⁶ The one-step synthesis is illustrated in Fig. 2. The structure of the product was confirmed by using UV, IR, NMR, MS/MS, and HPLC analyses. The isotopic purity of acetaldehyde-d₄ used for the synthesis was >98 atom % D according to Sigma-Aldrich. The isotopic purity of SAL-d₄ synthesized was found to be >97% based on the relative abundance of *m/z* 184 to the combined abundance of ions *m/z* 180, 181, 182, 183, and 184 in the MS spectrum of purified SAL-d₄. (+)-(*R*)/(-)-(*S*)-SAL enantiomers were prepared from racemic SAL as described previously.¹⁷ Milli-Q (Millipore Corp., Bedford, MA, USA) water was used throughout the work.

HPLC/ESI-MS/MS

The system consisted of two pumps (LC-10ADvp, Shimadzu, Toyoto, Japan), an autoinjector equipped with a 20 μL sampling loop (SIL-10A, Shimadzu), an on-line degasser (DGU-12A, Shimadzu), and an ion trap mass spectrometer with an ESI source (LCQ Deca, ThermoFinnigan, San Jose, CA, USA). Both the HPLC system and the mass spectrometer were controlled by Xcalibur software (ThermoFinnigan).

A β -cyclodextrin-bonded chiral HPLC column (250×2.1 mm, 5 μm , CYCLOBOND I 2000; Astec, Whippany, NJ, USA) connected to a C_{18} guard column (20×2.1 mm, 5 μm) was used for the separation. Column temperature was maintained at 4°C. Isocratic elution was used with 150 mM ammonium acetate buffer (pH 5.5) as mobile phase at a flow rate of 0.20 mL min^{-1} . A flow splitter was placed prior to the MS detector to divert approximately 90% of eluent to waste. The flow rate of eluent passing through the sampling capillary into the ESI-MS system was actually measured to be $\sim 20 \mu\text{L min}^{-1}$.

The mass spectrometer was operated in positive ion mode. Multiple mass spectrometry (MS/MS) experiments were performed to isolate and fragment the targeted ions. The operating conditions of the MS detector were optimized with a solution of SAL (5 $\mu\text{g mL}^{-1}$) infused into the ESI-MS system with a syringe pump at a flow rate of 10 $\mu\text{L mL}^{-1}$. Maximum signal intensity of the $[\text{M}+\text{H}]^+$ ion was obtained under the following conditions: sheath gas flow, 40 arbitrary units; auxiliary gas, 0 arbitrary units; capillary temperature, 220°C; spray voltage, 2.0 kV. For the MS/MS experiments, the relative collision energy used was 30% with an isolation width of 1.0 u. The other parameters were optimized using the Autotune Program.

Preparation of foodstuff samples for SAL enantiomer analysis

Dried banana chip and grape seed samples were collected from local grocery stores and ground. Weighed portions (1.00 g each) of a sample were suspended in 5.00 mL ice-cold 0.1 M HCl solution and (*R/S*)-SAL- d_4 was added at 1.00 $\mu\text{g mL}^{-1}$ as an internal standard. The suspension was homogenized with a model 550 sonic dismembrator (Thermo-Fisher Scientific) for 5 min on ice and left on a shaker for 30 min. The homogenate was centrifuged at 8000 rpm for 10 min at 4°C. The supernatant was collected. Ice-cold ethanol (5.00 mL) was added to precipitate proteins. The mixture was left on ice for 30 min before being centrifuged at 8000 rpm for 10 min at 4°C. The supernatant was collected and filtered through a 0.45 μm membrane filter. Portions (20 μL each) of the filtrate were injected into the HPLC/ESI-MS/MS system for analysis.

RESULTS AND DISCUSSION

HPLC separation of SAL enantiomers

Although separations of SAL enantiomers on cyclodextrin-bonded chiral stationary phases (CSP) have been reported previously,^{19,20} these separations used mobile phases containing non-volatile components such as phosphate buffer that are not compatible with MS detection. One recently published work described a chiral HPLC/MS separation of SAL enantiomers on a β -cyclodextrin (β -CD)-bonded column.²⁵ In the present work, we made a thorough study on the enantiomeric separation of SAL enantiomers involving testing several CSPs and mobile phases that were MS detection friendly. The tested CSPs included cellulose derivative-coated, macrocyclic glycoprotein-bonded, chiral crown ether-bonded, Pirkle-type, and β -CD-bonded silica particles. Results indicated that β -cyclodextrin-bonded CSP was most promising for resolving SAL enantiomers. Therefore, an optimum solvent system containing only volatile components was investigated for this CSP. Cyclodextrin-bonded CSPs have been shown to separate chiral compounds in three different modes, reversed-phase, normal-phase, and the

polar organic mode. As SAL enantiomers are highly hydrophilic, normal-phase mode was not a choice for their separation. Tests in the polar organic mode failed due to low retention of the solutes on the CSP. Therefore, efforts to separate the enantiomers were focused on using reversed-phase mode. It is common to use either acetonitrile or methanol as an organic modifier under reversed-phase conditions for improved peak shapes. However, it was found that retention times of SAL enantiomers decreased dramatically with the addition of these organic modifiers and the enantiomeric resolution was compromised significantly. Therefore, no organic modifier was added to the mobile phase. After comparatively studying four aqueous buffer systems prepared from ammonium acetate, ammonium formate, ammonium trifluoroacetate, and triethylammonium acetate, ammonium acetate buffer solution was chosen as mobile phase. Further, buffer concentration and pH value were studied. For the optimum separation results, a mobile phase consisting of 150 mM ammonium acetate buffer at pH 5.5 was deployed. A typical chromatogram from separating a racemic SAL sample is shown in Fig. 3(a). Under these conditions, resolution of SAL enantiomers was achieved with a resolution value of 0.6 ($R=2(t_R^2 - t_R^1)/(W_1+W_2)$). The peaks were identified by comparing the retention times with those of authentic (-)-(S)-SAL and (+)-(R)-SAL enantiomers. As can be seen, (-)-(S)-SAL was eluted before (+)-(R)-SAL. Since the enantiomeric resolution was not adequate enough for analysis of any real-life samples, further studies were carried out to improve it. It was found that the retention times of SAL enantiomers on a β -CD-bonded chiral column could be substantially increased by lowering the column temperature, thus leading to a much improved resolution of this pair of enantiomers, as shown in Fig. 3(b) (R value increased from 0.6 to 1.1).

ESI-MS/MS of SAL and SAL-d₄

The ion-trap MS detector used in this work could be operated in either negative ion mode or positive ion mode. Under the optimized chiral HPLC conditions, detection of SAL enantiomers in negative ion mode was found to be far less sensitive than in positive ion mode. Moreover, tandem mass spectrometry (MS/MS) experiments were performed to improve detection specificity. For the purposes of studying the ESI collision-induced dissociation (CID) of SAL as well as its quantification by isotope dilution HPLC/MS/MS, a deuterium-labeled analog of SAL (salsolinol- $\alpha,\alpha,\alpha,1$ -d₄, SAL-d₄) was synthesized. The synthetic route and its chemical structure are illustrated in Fig. 2. The ESI CID spectra of SAL and SAL-d₄ are shown in Fig. 4. As can be seen, the protonated molecule ions $[M + H]^+$ at m/z 180 and 184 were detected for SAL (Fig. 4(a)) and SAL-d₄ (Fig. 4(b)), respectively. Product ions of SAL were observed at m/z 163, 145, and 137. The structures of these fragments are proposed and shown in Fig. 5 (a). To a certain degree, these structural elucidations were supported by the CID spectrum of SAL-d₄. For each of the three fragment ions, there were one or more corresponding fragments containing deuterium atoms formed from SAL-d₄ (Fig. 5(b)). For example, ion $C_8H_9O_2^+$ m/z 137 was observed in both SAL and SAL-d₄ CID mass spectra because it does not contain any deuterium atoms. In corresponding to ion $C_{10}H_{11}O_2^+$ m/z 163 from the loss of ammonia (m/z 180 $\rightarrow$ m/z 163) in SAL ion $C_{10}H_9^2H_2O_2^+$ m/z 166 was formed from SAL-d₄ according to the fragmentation pathway proposed (shown in Fig. 5(b)). It is worth noting that the fragment ions in the CID spectrum of SAL-d₄ (Fig. 4(b)) indicate there was hydrogen scrambling in the fragmentation. In addition to the equilibrium between the ions described in the proposed fragmentation pathway (Fig. 5(b)), other causes may also be responsible for the hydrogen scrambling such as the proton/deuteron exchange between $[SAL-d_4+H]^+$ or 2H -containing ions and protic solvent molecules in the ion trap. Although several other fragmentation pathways can be speculated from the results presented for the CID of SAL and SAL-d₄, further studies including using a triple quadrupole instrument (to obviate the possible solvent exchange problems) as well as multiple deuterium-labeled SAL analogs such as SAL-d₁ and SAL-d₄ are needed to confirm the pathways proposed. It is most important that CID of both $[M+H]^+$ ions

of SAL and SAL-d₄ produced prominent product ions m/z 163 for SAL and m/z 166 for SAL-d₄, respectively. This allowed sensitive and specific quantification of SAL enantiomers by using isotope dilution HPLC/MS/MS.

Use of a stable isotope labeled analyte analog as internal standard is generally advantageous in any quantitative mass spectrometric method. It is usually considered to be essential in order to correct for matrix effects. Because the physico-chemical properties of the isotope-labeled internal standard are similar to those of analytes, the ratio of signal intensities for analyte/internal standard is ideally independent of the recoveries from chemical processes such as sample extraction and the degree of ionization in ESI, therefore providing a reliable basis for quantification. SAL-d₄ was chosen as the internal standard. From the HPLC/ESI-MS/MS chromatograms shown in Figs. 3(b) and 3(c), SAL and SAL-d₄ were co-eluted indicating they had identical chromatographic behavior. In addition, based on the equal peak areas/heights seen from these chromatograms these two compounds had the same MS/MS detection response factor. Therefore, it was reasonable to expect that the use of SAL-d₄ as internal standard corrected any effects on the HPLC/ESI-MS/MS quantification from potential variations in extraction efficiency, ionization, and MS/MS detection.

Quantification of (*R/S*)-SAL enantiomers

Using m/z 180 $\rightarrow$ 163 for (*R/S*)-SAL enantiomers and m/z 184 $\rightarrow$ 166 for (*R/S*)-SAL-d₄ enantiomers (internal standard) in selected reaction monitoring (SRM) MS/MS mode, the quantification was carried out by means of the signal ratio of analyte to internal standard. Five-point calibration curves were prepared with authentic racemic SAL solutions at concentrations ranging from 100 ng mL⁻¹ to 2 μ g mL⁻¹ racemic SAL. Linear calibration curves were obtained for both enantiomers with regression ($r^2 > 0.998$). The relative response factors of SAL/SAL-d₄ determined by the slope of individual calibration curves were 6.3 for the (-)-(*S*)-SAL enantiomer and 5.9 for the (+)-(*R*)-SAL enantiomer, respectively. From the calibration curves, the limit of detection was estimated to 15 ng mL⁻¹ for each SAL enantiomer (signal/noise ratio = 3). The precision of the assay was evaluated by repeatedly analyzing a mixture of authentic (*R/S*)-SAL at 250 ng mL⁻¹ and (*R/S*)-SAL-d₄ (internal standard) at 1 μ g mL⁻¹. Intraday relative standard deviation (RSD) was found to be 1.4% ($n = 5$), and interday RSD was 3.6% ($n = 5$ in 10 days). Further, recovery of SAL from sample matrix was investigated by comparing signal abundance of (*R/S*)-SAL-d₄ spiked into an extract of dried banana chips with that from an (*R/S*)-SAL-d₄ standard aqueous solution. The recovery was estimated to be $100.5 \pm 5.3\%$ ($n = 6$).

Enantiomeric determination of SAL in foodstuff samples

The present chiral HPLC/MS/MS method was tested by quantifying SAL enantiomers in two foodstuff samples, i.e. dried banana chips and grape seeds. Typical extracted ion chromatograms obtained from analyzing these samples are shown in Fig. 6. In all the three samples of dried banana chips, both SAL enantiomers were detected. The concentrations of (*R*)-SAL and (*S*)-SAL were determined to be 481.3 ± 20.5 ng g⁻¹ tissue and 502.2 ± 14.5 ng g⁻¹ tissue (mean $\pm$ SD, $n = 3$), respectively. It's worth noting that (*R*)-SAL and (*S*)-SAL concentrations found in dried banana chips by the present chiral HPLC/MS/MS method were about 100 times lower than those (~ 50 μ g g⁻¹ tissue) measured by chiral HPLC methods with electrochemical detection (ECD).^{27,28} This might be due to two factors: (1) the samples of dried banana chips were collected at different locations and different times, and (2) the HPLC/ECD methods lacked sufficient specificity for the determination. It is worth noting that all the analytical results indicated that the enantiomeric ratio of (*S*)-SAL to (*R*)-SAL was close to 1:1. Although low levels of SAL were detected in wines,²⁸ SAL was not detected in all the three grape seed samples tested in this work. The analysis also revealed that dried banana chips and fresh bananas contained dopamine at high levels, but grape seeds did not. These data will be reported later.

CONCLUSIONS

A stable isotope dilution HPLC/MS/MS method was developed for simultaneous determination of individual salsolinol enantiomers. The study showed that this pair of enantiomers was most efficiently resolved on β -cyclodextrin-bonded chiral stationary phase with an MS detection-friendly mobile phase. Under the experimental conditions, ESI-MS/MS detection of SAL enantiomers was highly sensitive and specific. Using the present method, foodstuff samples including dried banana chips and grape seeds were analyzed to determine SAL enantiomers requiring minimum sample pretreatment and no pre-column derivatization. It was found that both (*R*)-SAL and (*S*)-SAL enantiomers were at a level of 500 ng g⁻¹ tissue in dried banana chips with an enantiomeric ratio close to 1. No SAL was detected in the three samples of grape seeds analyzed.

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Figure 1.

Chemical structures of MPTP, salsolinol, and *N*-methysalsolinol. *N*-Methysalsolinol exhibits some neurotoxicological properties similar to those of MPTP.

Figure 2.

Synthesis of salsolinol- $\alpha,\alpha,\alpha,1$ -d₄ (SAL-d₄) used for the study of CID fragmentation and for stable isotope dilution HPLC/MS/MS quantification of salsolinol.

Figure 3.

Resolving SAL enantiomers by chiral HPLC/MS/MS: temperature effects. Extracted ion chromatograms of m/z 180 $\rightarrow$ 163 of (R/S)-SAL from separations at (a) 20°C, (b) 4°C, and (c) m/z 184 $\rightarrow$ 165 of (R/S)-SAL-d₄ from a separation at 4°C.

Figure 4.
ESI-MS/MS spectra of $[M+H]^+$ ions of SAL (a) and SAL-d₄ (b).

Figure 5.
Proposed CID fragmentation pathways of SAL (a) and SAL- d_4 (b).

Figure 6. HPLC/ESI-MS/MS determination of (*R*)-SAL and (*S*)-SAL enantiomers in foodstuff samples: HPLC/MS/MS extracted mass chromatograms of m/z 180 $\rightarrow$ 163 from analyzing (a) dried banana chips and (b) grape seeds; (c) HPLC/MS/MS extracted mass chromatograms of m/z 184 $\rightarrow$ 166 for SAL-d₄, the internal standard.