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In Vitro and *in Vivo* Metabolism of Salsolinol, an Endogenous Isoquinoline Neurotoxin, in Rats

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ABSTRACT : **Salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, SAL), a dopaminergic isoquinoline neurotoxin, has been implicated to contribute the etiology of Parkinson's disease and neuropathology of chronic alcoholism . In our previous results, SAL was reported to have the mutagenicity and clastogenicity not in bacteria but in mammalian cells, and its genotoxic potential was known to be potentiated in the presence of rat liver S-9 fraction. This may indicate that some metabolite(s) of SAL was involved in the mutagenic potentials. To investigate the SAL metabolites, the metabolism studies of SAL were conducted *in vitro* rat liver S-9 fraction and *in vivo* using rats by high performance liquid chromatography and gas chromatography/mass spectrometry. The methylated metabolite of SAL was found in urine of rats, while the same methylating form of metabolite was not produced from the *in vitro* metabolism system using rat liver S-9 fraction.**

Keywords : **Salsolinol, *in vitro* metabolite, metabolism, rats, high-performance liquid chromatography, gas chromatography/mass spectrometry**

Introduction

Salsolinol (SAL; 1,2,3,4-tetrahydro-1-methyl-6,7-isoquinolinediol) was reported as an endogenous alkaloid derived from the nonenzymatic Pictet-Spengler condensation of neurotransmitter dopamine with acetaldehyde *in vitro* during alcohol metabolism (Yamanaka *et al.*, 1970).

This compound is produced in the brain by condensation of dopamine with pyruvic acid, its subsequent decarboxylation and reduction (Dostert *et al.*, 1990). Tetrahydroisoquinoline derivatives have been extensively studied because not only they have been found in the brain of postmortem specimens of patients with Parkinsonism, but also they are able to induce Parkinsonian symptoms in rats (Collins *et al.*, 1975). The SAL was also found in certain beverages and foodstuff such as soy sauce, beer and bananas (Riggin *et al.*, 1976). Parkinsonisms are reportedly related to SAL in rats (Naoi *et al.*, 1996). Our laboratory also reported (Ryu *et al.*, 1999a; 1999b; Jung *et al.*, 2001) the genotoxic spectra of SAL. Analysis of these catecholamine analogues has been performed by high-performance liquid chromatography (HPLC) with electrochemical detection and gas chromatography/mass spectrometry (GC/MS; Odink *et al.*, 1986; Sasaoka *et al.*, 1988; Naoi *et al.*, 1988; Pianezzola *et al.*, 1989; Dufay *et al.*, 1991; Sjoquist *et al.*, 1986).

In this study, the detections of metabolites from *in vitro* metabolism system and the collected urine of rats administered SAL were performed by using HPLC and GC/MS, respectively. Part of these has already been presented in the preliminary study (Rhee *et al.*, 1999; Ryu et al., 1999b).

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Materials and Methods

Reagents

Salsolinol and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) were purchased from Sigma (St. Louis, USA). Sodium 1-heptane sulfonate, an ion-pair reagent, was purchased from Fluka Chemie (Buchs, Switzerland). *N*-Methyl-*bis* (trifluoroacetamide) (MBTFA) was purchased from Pierce (Rockford, Illinois, USA). The other chemicals were obtained from Junsei (Tokyo, Japan) and Kanto (Tokyo, Japan). All other solvents were of HPLC grade from J. T. Baker (Phillipsburg, USA).

Animals

Male Sprague-Dawley rats (8 weeks-old, 250±10 g) were purchased from Daehan Laboratory Animals (Eumseong, Chungbuk, Korea) and housed in an air-conditioned room having a 12/12 hour light/dark cycles. Food pellets purchased from Samyamg (Seoul, Korea) and tap water were available *ad libitum* to rats that were acclimatized to the

animal facility for 1 week before use.

Preparation of liver microsomal fraction (S9) and 4% S9 mixture

The preparation of rat-liver S-9 fraction and 4% S9 mixture for metabolism study was previously reported (Ames *et al.*, 1973; Maron and Ames, 1983). The S-9 fraction was stored immediately at −80°C before use.

In vitro metabolism study using rat liver S9 mixture

Two hundred fifty μl of 46.4 μM SAL solution (100 μg SAL/ 0.1 M perchloric acid 10 ml) was added to 25 ml of S9. The mixture was incubated at 37°C water bath for 0, 0.5, 1, 2, 3, 5, 10 and 24 hrs. The aliquot of 0.8 ml of the incubated sample was mixed with 0.1 ml of 1 M perchloric acid containing 0.4 mM sodium metabisulfite and 0.1 mM disodium EDTA. The mixture was centrifuged at 3000 rpm for 10 min at 4°C. 0.9 ml of the supernatant was transferred to a tube and 0.1 ml of 1 M perchloric acid solution containing 0.4 mM sodium metabisulfite and 0.1 mM disodium EDTA was added. The mixture was centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was filtered through a 0.22 μm nylon membrane filter (Millipore, USA).

In vivo metabolism in urine of rats

The SAL (50 mg/kg×2, 2 hr interval) was intraperitoneally administered to rats. The urine sample was collected from a metabolic cage for 48 hr. The urine sample (1 ml) was mixed with 0.1 ml of 1 M perchloric acid containing 0.02% sodium metabisulfite and 0.01% disodium EDTA. To remove the protein, the mixture was centrifuged at 3000 rpm for 10 min at 4°C, and the supernatant was incubated for 30 min at 80°C to hydrolyze SAL conjugates. Phenylboronic acid solid phase (PBA) cartridges (Varian, Harbor City, CA, USA) were used for the solid-phase extraction of SAL. The cartridges were conditioned by washing twice with each 1 ml of methanol, water and saturated $NaHCO_3$ buffer solution (pH 9). The pH value of the sample was adjusted to 6.5-7 by adding 1 M KOH, and the sample was loaded to to the PBA cartridges and passed through by gentle low-pressure aspiration. After washing twice with 0.2 ml of water and 0.4 ml of methanol, the adsorbed SAL was eluted from the cartridge with 0.1 ml of 5 M formic acid-methanol (1:5). The formic acid-methanol fraction was evaporated to dryness. The residue was derivatized with 100 μl of MSTFA (30 min, 70°C), and consecutively 10 μl of MBTFA (20 min, 80°C). 2 μl of the reaction mixture was injected into GC/MS.

HPLC apparatus and chromatographic conditions

The HPLC system was composed of a electrochemical detector (Model 460, Waters; set at 400 mV), 616 Pump and 600S Controller. The separation was performed using a reverse phase Lichrosorb RP18 (5 μm, 4.6 mm×15 cm). The mobile phase was consisted of 25 mM sodium phosphate buffer (pH 3.0) containing 0.2 mM sodium 1-heptane sulfonate and 3% acetonitrile, and the flow rate of column was 1.0 ml/min. 50 μl of the sample was injected to HPLC. The detection limit of SAL was about 25 ng/ml.

GC/MS apparatus and chromatographic conditions

The GC/MS system was composed of GC/MSD (HP5890/ HP5970), HP7673 Autosampler, and MS ChemStation. A crosslinked 5% phenylmethylsiloxane capillary column (25 m length×0.2 ID×0.33 μm film thickness) was used. High purity of helium was used as the carrier gas. The splitless injection mode was used; the purge valve was turned on 0.5 min after injection, with flow rate of 0.8 ml/min. The injector and detector temperatures were maintained at 250°C and 300°C, respectively. The GC oven temperature was held at 50°C for 1 min, and increased to 300°C by 10°C/min, and finally stayed for 26 min at 300°C.

Results and Discussion

In vitro metabolism experiment was conducted using 4% S9 mixture by the analytical method using HPLC with an

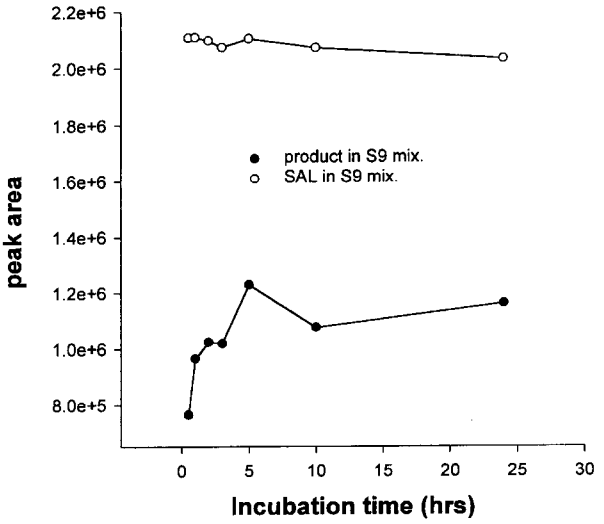

Fig. 1. Effect of various incubation times on the production of *in vitro* salsolinol (SAL) metabolite. Salsolinol (464 nM) was added to 25 ml of reaction mixture including 4% S9 fraction of rat liver microsome.

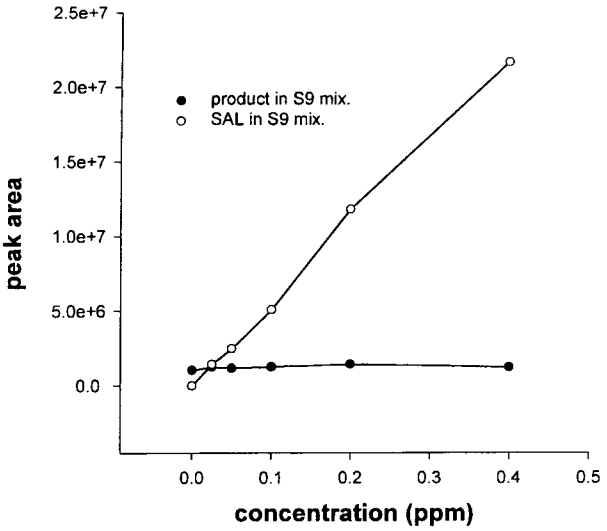

Fig. 2. Effect of various concentrations of salsolinol (SAL) on the production of in vitro salsolinol metabolite. Theses reaction mixture was incubated for 3 hr at 37°C.

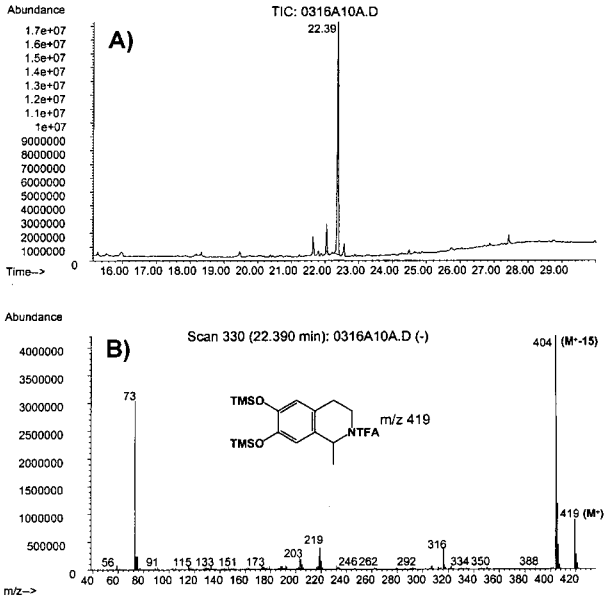

Fig. 3. Total ion chromatogram (A) and scan mass spectrum (B) of the authentic salsolinol.

electrochemical detector commonly applied to quantification of catecholamine analogues as described in the experimental section. Various incubation times (0-25 hrs) was not affected significantly the production of SAL metabolite in tubes containing 464 nM of SAL and 4% S9 mixture (Fig. 1). No dose-dependent effect of SAL on the formation of SAL metabolite was observed at 3 hrs of incubation time (Fig. 2). This *in vitro* experiment indicates that SAL metabolite was not produced in the given conditions using 4% S9 mixtures.

In vivo experiment using rats for identification of the metabolite of SAL, the solid phase extraction by phenylboronic phase (PBA) cartridge and two step drivatization by *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) and *N*-methyl-*bis*(trifluoroacetamide) (MBTFA) were applied for GC/MSD analysis of dopaminergic agents with hydroxyl and amine group.

The total ion chromatogram and mass spectrum of SAL-O-TMS and -N-TFA derivative were shown in Fig. 3A and 3B, respectively. The molecular ion of the SAL derivative with 22.4 min of the retention time is m/z 419, and the other characteristic ions are m/z 73 and m/z 404 (M^+-15).

One metabolite of SAL in urine 48 hrs after the intraperitoneal administration of SAL was confirmed to be the methylate of SAL which showed the characteristic molecular ion of m/z 361 as shown in the mass spectrum of Fig. 4C. The metabolite was interpreted as a structure which trimethylsilyl group on 2 or 3 position of SAL was substituted by a methyl group. The other characteristic ion was

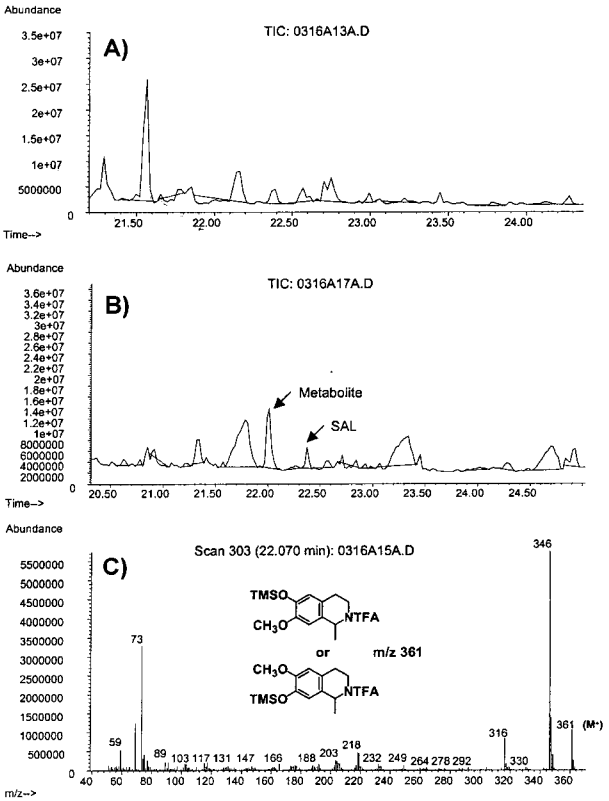

Fig. 4. Total ion chromatograms obtained from blank urine (A) and from urine sample after 48 hr (B), and the scan spectrum of the methylated metabolite of salsolinol in urine sample after 48 hr (C). The methylated metabolite has the retention time of 22.07 min and the characteristic ion for the molecular weight of salsolinol-TMS was m/z 361.

m/z 346 (M^+-15). Small amount of parent SAL peak was detected in urine of rats 48 hrs after the administration of SAL, indicating that SAL may be biotransformed rapidly within 48 hrs.

In summary, SAL metabolism studies were conducted *in vitro* and *in vivo*. The methylating form of SAL was found in urine of rats, while the same form of metabolite was not produced *in vitro* metabolism system using rat liver S-9 fraction.

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