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Journal of Chromatography B, 735 (1999) 299–303

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Short communication

Quantitative determination of endogenous tetrahydroisoquinoline salsolinol in peripheral blood mononuclear cells by gas chromatography–mass spectrometry

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Received 13 October 1998; received in revised form 15 September 1999; accepted 17 September 1999

Abstract

Endogenous 1-methyl-1, 2, 3, 4-tetrahydro-6,7-dihydroxyisoquinoline (salsolinol) could be a potential marker involved in the etiology of alcoholism. The amount of salsolinol analyzed previously from plasma and urine by different methods depends on several dietary conditions because nutrition has an important influence on salsolinol excretion. Whereas plasma salsolinol is influenced by the diet the salsolinol from peripheral mononuclear cells should be endogenously formed. Therefore, a method for the quantification of *S*-and *R*-salsolinol from lymphocytes by using gas chromatography–mass spectrometry was developed. The average amount of salsolinol in 10^6 cells was 1.25 ng corresponding to 2.41×10^{-5} *M* and was shown to be much higher than the plasma salsolinol concentration (2.6×10^{-9} *M*).

Keywords: Salsolinol; Tetrahydroisoquiholiner

1. Introduction

Evidence has been obtained that dopamine and other catecholamines are present in single lymphocytes by use of capillary electrophoresis with electrochemical detection [1].

Tetrahydroisoquinolines (TIQs) are condensation products of catecholamines and their analogs with aldehydes or pyruvate following enzymatic or non-enzymatic reaction. The amount of TIQs found in body fluids is considered to be associated with some diseases such as diabetes, Parkinsonism, phenylketonuria, and alcoholism [2–5]. Dopamine-derived TIQs appear to have a variety of effects on dopaminergic neurons such as the stimulation of the release of stored catecholamines and the inhibition of catecholamine reuptake. They are also inhibitors of monoamine oxidase (MAO), catechol-*O*-methyltransferase (COMT), and tyrosine hydroxylase [6–8]. One of the TIQ compounds is the dopamine-derived alkaloid salsolinol (SAL) (Fig. 1). Several reports suggest an involvement of SAL and other TIQs in the biochemical mechanisms underlying alcohol

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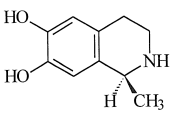
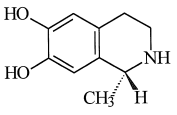
0378-4347/99/$ – see front matter
PII: S0378-4347(99)00431-4



S-SAL



R-SAL

Fig. 1. Chemical structure of *R*- and *S*-salsolinol (*R*- and *S*-SAL).

addiction [4,5,9]. It has been assumed that the condensation of dopamine with pyruvate followed by oxidative decarboxylation and reduction forms SAL. Another pathway for SAL formation is the condensation of dopamine with acetaldehyde (Pictet–Spengler reaction), the metabolite of alcohol. Therefore, it might be possible that SAL contributes to the biochemical and behavioral effects of alcoholism by affecting catecholamine function in the peripheral as well as in the central nervous system. Otherwise, it is now well established that SAL is excreted not only by alcoholics, but also by nonalcoholic (control) subjects indicating the existence of normal endogenous SAL sources [4,5].

SAL is also present in some food and alcoholic beverages such as beer and wine [10]. As far as the relationship between alcohol consumption and SAL formation is concerned, the results from previous work are contradictory. The variability in the reported levels of SAL might be a result of variables, including dietary conditions during the experiments or the duration of ethanol ingestion and analytical problems associated with the detectability of the TIQs. We reported plasma and urine SAL levels in humans [4] and found that nutrition has an important influence on SAL excretion.

Therefore, experiments were undertaken to use biological material for SAL analyses which is less influenced by nutritional factors. Based on the discovery of endogenous dopamine, the precursor of SAL, in lymphocytes by Berquist in 1994 [1], we used peripheral blood mononuclear cells (PBMC) for the quantification of SAL. We report here that PBMCs of healthy subjects contain SAL which could be quantified by GC–MS. Furthermore, we were able to separate the enantiomers of SAL by the method we described previously [11].

2. Materials and methods

2.1. Chemicals

The enantiomers of SAL were synthesized as described previously [11]. Racemic deuterated SAL was prepared as internal standard by treating 2, 5, 6-[$^{2}H_{3}$]dopamine (obtained from IC Chemikalien, Munich, Germany) with acetaldehyde [12]. *R*-(−)-2-Phenylbutyryl chloride was synthesized by reaction of *R*-(−)-2-phenylbutyric acid with thionyl chloride [13]. A 0.1 *M* solution was used for the derivatization of SAL. *N*-Methyl-*N*-trimethyl-silyltrifluoroacetamide (MSTFA) was purchased from Macherey Nagel (Düren, Germany). Phenylboronic acid solidphases (PBA) were supplied by Varian (Harbor City, CA, USA).

2.2. Preparation of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were isolated as described previously in detail [14]. In brief, PBMC were isolated from the leukapheresed blood of normal individuals after a Ficoll–Hypaque gradient centrifugation and exposed to countercurrent centrifugal elutriation using a Beckman system (Beckmann Instruments, Fullerton, CA). Elutriated monocytes were 99% viable as determined by trypan blue exclusion, and 96% were positive for the monocyte markers Leu-M3 (Beckton Dickinson, Mountain View, CA) and MO2 (Coulter Immunology, Hialeah, FL). The coefficient of variation (C.V.) of counting 10^{6} cells was 3%. Aliquots of 1×10^{6} cells were collected in Eppendorf tubes and centrifuged 10 900 rpm for 1 min. The supernatant was removed and the tubes were used for the investigation of cellular uptake mechanism or stored at −80°C until used for the analyses of SAL.

2.3. Extraction of SAL from peripheral blood mononuclear cells

Extraction of SAL from definite amount of cells (4–10 x10^{6}) was done by addition of 1 ml of aldehyde trapping mixture (1 *M* perchloric acid,

containing 0.01% ethylene glycol-bis(β-aminoethyl ether)-*N*, *N*, *N′*, *N′*-tetraacetic acid (EGTA), 0.01% semicarbazide hydrochloride and 0.02% sodium metabisulfite). The samples were centrifuged at 1000 *g* for 10 min at 0°C. The supernatant was used for the extraction procedure. A definite amount (1 ng/ml supernatant) of deuterated standard was added. One hundred-ml PBA cartridges were used for the solid-phase extraction of SAL. The cartridges were conditioned by washing twice with 1 ml of methanol, 1 ml of water, and 1 ml of saturated $NaHCO_3$ buffer solution (pH 9). Samples were adjusted to pH 9 with NaOH (1 mol/l) and saturated $NaHCO_3$ solutions and were loaded on PBA cartridges and passed through by gentle low-pressure aspiration. After washing twice with water (1 ml) and methanol (2 ml), the adsorbed TIQs were eluted from the cartridge with 1 ml of MeOH that was saturated with HCl gas. The sample was evaporated to dryness and derivatized with 100 μl of MSTFA (30 min at 70°C). Then, 100 μl of *R*-(−)-2-phenylbutyrylchloride was added, and the mixture was shaken for 1 min. The derivatized samples could be kept at 4°C for several days. Two μl of the reaction mixture was injected into the gas chromatographic–mass spectrometric system (GC–MS).

2.4. Calibration curves

Standard curves were prepared for each SAL enantiomer by adding varying known amounts (0.250–5 ng) of *R*- and *S*-SAL, respectively, and a fixed amount, 5 ng, of the deuterated SAL to 5 ml of water and carrying out the solid-phase extraction described above in triplicate. The derivatized samples were evaporated to dryness under a stream of dry nitrogen. The residue was dissolved in 20 μl of dry chloroform, and a 2-μl aliquot was immediately subjected to GC–MS analysis (using the selected-ion monitoring (SIM) mode). Standard curves were generated by least-squares linear regression analysis.

2.5. Studies of artifactual condensation and hydrogen–deuterium exchange, and recovery

A 5-ng amount of deuterated dopamine was added to the supernatant of lymphocyte extract (10×10^6 cells), and the PBA extraction procedure was carried out as described above. The samples were analyzed by GC–MS for the presence of deuterated SAL.

To ascertain that the deuterated standard was stable in an aqueous medium and that there was no H–D exchange during the sample preparation, deuterated SAL (10 ng) was added to 10 ml of aldehyde trapping mixture and the sample was worked up as described and was analyzed for the presence of non-deuterated SAL.

To determine the recovery and the limit of quantification of SAL, the pH of the cell solution (1×10^6 cells) was brought to pH 12 and it was allowed to stand for 24 h to remove any original SAL. After the pH had been adjusted to pH=7, 200 pg of deuterated SAL and different amounts (50, 100, 150 and 200 pg) of *R*-SAL and *S*-SAL were added to cell extracts (1 ml, 10^6 cells) and the samples ($n=5$) were worked up as usual and analyzed by GC–MS.

2.6. Investigation of cellular uptake mechanism and in vivo synthesis of SAL

4×10^6 cells were resuspended in 1 ml of Dulbecco's modified eagle's cell medium and were incubated for 1 h at 37°C with 0.5 ml of a solution of SAL (2.5 μ*M*) or dopamine (2.3 μ*M*) in cell medium with 100% humidity and 5% CO_2 in air in an Cellstar incubator. After centrifugation the pellets were washed five times with cell medium. The cells were worked up for the extraction of SAL and dopamine as described above. For the derivatization, the extracts were treated with MSTFA (1 h, 60°C) and then with MBTFA.

2.7. Instrumentation and chromatographic conditions

GC–MS resolution of TIQ enantiomers was carried out with a FISONS TRIO 1000 GC–MS data system. Derivatized samples were analyzed using a 20-m BGB-silaren capillary column (0.32 mm I.D. and 0.12-μm film thickness) from Chromtech (Hofheim am Taunus, Germany). Ultrapure helium was used as the carrier gas with a head pressure setting of 1 bar. The injector temperature was 300°C; interface and ion source temperatures were main-

tained at 300 and 250°C, respectively. The splitless injection mode was used; the purge valve was turned on 1.5 min after injection, with a split flow-rate of 25 ml/min during GC run. The GC oven temperature was held at 200°C for 2 min and then programmed at 10°C/min to 300°C for 15 min. Electron-impact ionization mass spectra were recorded in the SIM mode. The ions monitored were m/z 454 for SAL, and 456 for the standard.

3. Results and discussion

A GC–MS chromatogram for an extract of 4×10^6 PBMCs, prepared from the blood of a healthy human, is shown in Fig. 2. Beside the peaks of the deuterated salsolinol (internal standard) the chromatogram shows two peaks for SAL, the *S*- and *R*-SAL. This separation was achieved by the derivatization of the sample with a chiral reagent [12]. The analysis of human PBMCs revealed that SAL occurs as a racemic mixture of both enantiomers.

The correlation coefficients obtained for the calibration curves were consistently higher than 0.99. Two to 4×10^6 cells (from about 2-4 ml blood) were used for the SAL quantification from human PBMCs. The cellular concentration of SAL in the PBMCs was determined from the blood of six healthy subjects. The average amount of SAL in 1×10^6 cells was 1.25 ±0.32 ng (mean±S.D.). If the volume of a single PBMC is approximately 230 fl, [15] the SAL concentrations calculated from the average total moles for the PBMCs (5.5 amol) correspond to 2.42×10^{-5} M.The coefficients of variation (C.V.) obtained from the repeated analysis of 4×10^6 PBMCs of one subject were always within 1–2%, both for the intra-day ($n=3$) and the inter-day ($n=3$) experiments. The limit of quantification, defined as the concentration for which the standard deviation of five determinations is within ±10% of the mean value and with a C.V. of 8%, was found to be 100 pg/ml of cell extract (lacking original SAL) for each added SAL enantiomer. Therefore, if the average amount of SAL is 1.25 ng/10^6 cells at least 10^5 cells are needed for a quantitative SAL determination.The limit of detection was found to be at least 50 pg/ml. The recovery of SAL from PBMCs as determined by adding different amounts of SAL to SAL-free cell extracts was higher (80%) than from plasma or urine (65%) [4]. This result is due to the higher sample purity of cell extracts in comparison to plasma or urine samples. The sample work up procedure did not lead to any racemization of the enantiomers. As it was proved previously [12] no

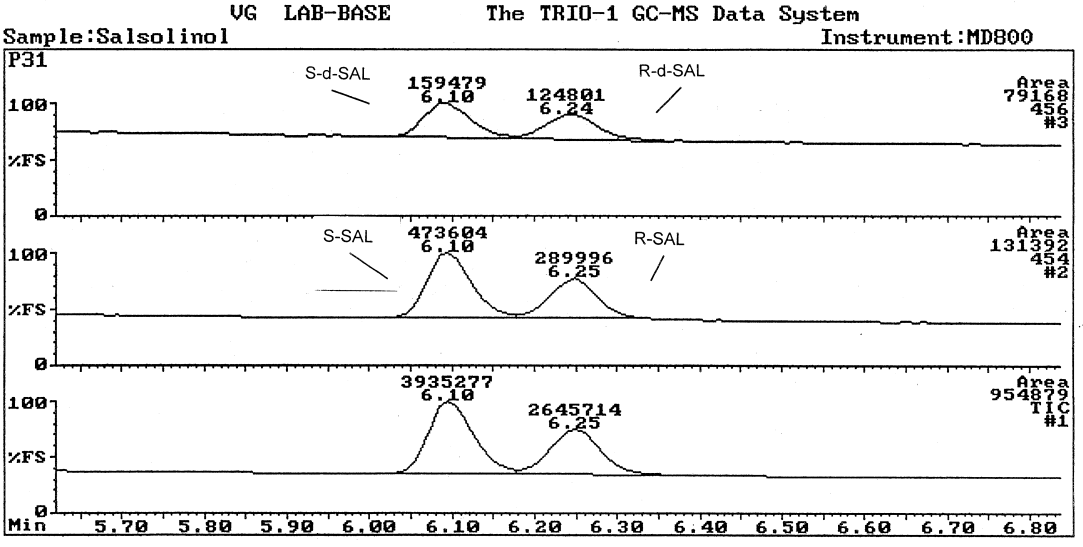


Fig. 2. GC–MS chromatogram of *R*- and *S*-salsolinol (*R*- and *S*-SAL, m/z 454) detected in lymphocytes using deuterated salsolinol (d-SAL, m/z 456) as internal standard.

SAL was formed after addition of deuterated dopamine to the acid cell extract. So, it was proved that there was no artificial formation of SAL from dopamine during extraction and assay. Deuterated SAL (internal standard) was found to be stable during the sample work up procedure.

Previously, we determined the plasma SAL concentration of healthy subjects and 0.466 ng SAL per ml plasma (2.6×10^{-9} *M*) was found. [4]. The present study shows that the SAL concentration in PBMCs is much higher (2.42×10^{-5} *M*) than the plasma SAL concentration.

In order to investigate if lymphocytes obtained directly from human blood may have accumulated SAL by active transport rather then by in vivo synthesis, the cells were incubated with racemic SAL for 1 h and the detected total amount of SAL was compared with untreated cells. The total SAL concentration was three-fold higher in the SAL treated cells in comparison to the untreated cells indicating that there might be an active uptake mechanism for SAL. However, the incubation of cells with dopamine (the precursor of SAL) led to a 16-fold higher total SAL concentration in the lymphocytes in comparison to the control cells. It has been demonstrated by capillary electrophoresis with electrochemical detection that there exists an active transport for dopamine and other catecholamines and that they are produced and stored by lymphocytes [1]. Our results demonstrate that salsolinol can be transported into the lymphocytes as well as synthesized by the lymphocytes themselves.

Because of the higher concentration of SAL in the cells in comparison to the plasma, determination of the SAL-concentration in PBMCs is apparently more powerful than from plasma.

Experiments will be undertaken to study the differences in SAL concentration of PBMCs from healthy subjects and alcoholics in order to explore whether the present method of determining SAL may help elucidate the etiology of alcoholism.

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