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Determination of free salsolinol concentrations in human urine using gas chromatography–mass spectrometry

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ABSTRACT

The urine concentrations of free salsolinol were determined in six healthy volunteers, using a gas chromatographic–mass spectrometric method with electron-capture negative-ion chemical ionization after derivatization with pentafluoropropionyl anhydride. The sensitivity of this method allows the quantification of salsolinol concentrations of 0.55 pmol/ml. The synthesis of [$^2H_4$]salsolinol from dopamine and [$^2H_4$]acetaldehyde via a Pictet–Spengler condensation is described; [$^2H_4$]salsolinol was used as the internal standard for salsolinol quantification. The urine concentrations of free salsolinol ranged from *ca.* 1 to 6 pmol/ml.

INTRODUCTION

Various dopamine-derived alkaloids have long been shown to be present in animal species and humans (for review see ref. 1). It has been suggested that changes in the levels of these alkaloids, those of tetrahydropapaveroline and salsolinol in particular, might underlie the addictive drinking of alcohol and cause or contribute to modifications in mental and/or neurophysiological states in different pathologies [2,3].

In healthy subjects, the presence of salsolinol (1-methyl-1,2,3,4-tetrahydro-6,7-isoquinolinediol) has been established in urine, cerebrospinal fluid and brain tissue using high-performance liquid chromatography coupled with electrochemical detection (HPLC–ED) or gas chromatography–mass spectrometry (GC–MS) after derivatization [4–10]. In these studies the limit of detection was, at best, 1 pmol/ml or 1 pmol/g.

Measurement of the daily urinary excretion of salsolinol has been suggested as one of the possible diagnostic methods for the early detection of Parkinson's disease [11]. However, the urinary concentration of salsolinol in healthy subjects under diet conditions excluding the intake of salsolinol-containing food or beverages showed great inter-individual variation [12–15]. In addition, the daily urinary output of salsolinol was found to be markedly lower in untreated parkinsonian patients than in healthy controls [8,16]. Therefore, further investigation of

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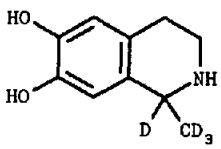

I

Fig. 1. Structure of [$^2H_4$]salsolinol (I).

salsolinol output for the early detection of Parkinson's disease requires an assay with the sensitivity required to measure accurately very low urine levels.

This paper describes a GC–MS method that allows quantification of urinary salsolinol concentrations as low as 0.55 pmol/ml. The urinary concentrations of free salsolinol in six healthy subjects are given, together with the synthesis of an appropriate deuterated standard (I, Fig. 1).

EXPERIMENTAL

Chemicals and reagents

(±)-Salsolinol hydrobromide was purchased from Roth (Karlsruhe, Germany). Phenylboronic acid (PBA) cartridges were from Analytichem International (Harbor City, CA, USA). Pentafluoropropionic anhydride (PFPA) was obtained from Pierce (Rockford, IL, USA). EDTA (disodium salt, dihydrated) was purchased from Sigma (St. Louis, MO, USA). [$^2H_4$]Acetaldehyde (CD_3CDO) and dopamine hydrobromide were from Aldrich (Milan, Italy). Sodium metabisulphite ($Na_2S_2O_5$), semicarbazide hydrochloride, tris(hydroxymethyl)aminomethane buffer and analytical-grade reagents were from Farmitalia Carlo Erba (Milan, Italy).

Preparation of 1-[$^2H_3$]methyl-1-[^2H]2,3,4-tetrahydro-6,7-isoquinolinediol hydrobromide (I)

To a solution of 3.97 g (17 mmol) of dopamine hydrobromide in 25 ml of ^2H-labelled water (D_2O) at room temperature, 1.0 g (21 mmol) of CD_3CDO was added and the solution was brought to pH 2 by adding D_2O. Air was replaced by nitrogen and the reaction vessel was closed. After standing at room temperature in the dark for seven days, the solution was evaporated to dryness maintaining the temperature below 30°C. After standing for 60 h at room temperature, the partly solid residue was treated with 5 ml of absolute ethanol and filtered, giving 1.64 g of a solid (m.p. 179–185°C). Two recrystallizations from 2-propanol afforded 0.94 g (21%) of I (m.p. 190–194°C). The product was shown to be free from unreacted dopamine by selected-ion monitoring (SIM) GC–MS, and to contain four deuterium atoms.

Urine collection and salsolinol extraction

Six healthy male volunteers aged 32.5 ± 3.7 years (mean ± S.D.) participated in the study. Intake of chocolate, fresh and dried bananas, soya sauce, beer and port wine, all known to contain appreciable amounts of salsolinol [9,12], was forbidden for 48 h before and throughout urine collection; alcoholic beverage intake was limited to a maximum of three glasses of wine per day.

The 24-h urines were collected in 1-l polyethylene containers previously stored at 4°C and containing 0.5 g of EDTA, 0.5 g of $Na_2S_2O_5$ and 0.5 g of semicarbazide hydrochloride. After every voiding the container was gently stirred and replaced in the refrigerator at 4°C. Upon completion of urine collection, urine samples were kept at −80°C until analysis.

A 1-ml volume of urine spiked with 17 pmol of deuterated standard (I) was brought to pH 8.4–8.5 with 1 *M* Tris buffer, and then loaded into a PBA cartridge previously conditioned by sequential washing with 1 ml of methanol, 1 ml of water, 1 ml of 0.1 *M* hydrochloric acid, 1 ml of 0.3% ammonium hydroxide and 1 ml of 0.01 *M* pH 8.5 $(NH_4)_2SO_4$ buffer (twice). After washing with 1 ml of water (three times) and 1 ml of methanol (twice), salsolinol was eluted with 0.5 ml of 1 *M* acetic acid in methanol (three times). After evaporation to dryness at 40°C under a nitrogen stream, the samples were derivatized for GC–MS analysis.

Derivatization procedure

The samples were treated with PFPA (25 μl) in anhydrous ethyl acetate (25 μl) at 70°C for 30 min. Immediately prior to injection, excess reagent and ethyl acetate were removed under nitrogen and the residue was redissolved in 50 μl of dry ethyl acetate. A 1-μl volume was injected into the gas chromatograph–mass spectrometer.

GC–MS instrumentation and instrumental conditions

A Finnigan MAT 4600 Superincos GC–MS data system instrument combined with a Varian 3400 gas chromatograph was used. The instrument was equipped with a 15 m × 0.53 mm I.D. DB-5 fused-silica megabore capillary column, film thickness 1.5 μm (J&W Scientific, Folsom, CA, USA) and a Varian septum-equipped programmable injector. Helium at a pressure of $69 \cdot 10^3$ Pa was used as carrier gas. An initial oven temperature of 75°C was held for 2 min and then raised to 280°C at 15°C/min. The on-column injector temperature was held at 58°C for 10 s then increased to 280°C at 140°C/min, with a final hold time of 5 min. The transfer-line temperature was 280°C, and the ion-source temperature was 180°C.

The ionization technique used was electron-capture negative-ion chemical ionization (EC-NICI) with isobutane as moderating gas at 200 Pa fore-pressure. The electron energy was 70 eV, the emission current was 0.30 mA, and other ion-source parameters were adjusted to give the maximum negative ion current in the mass range of interest, using perfluorotributylamine as a reference compound.

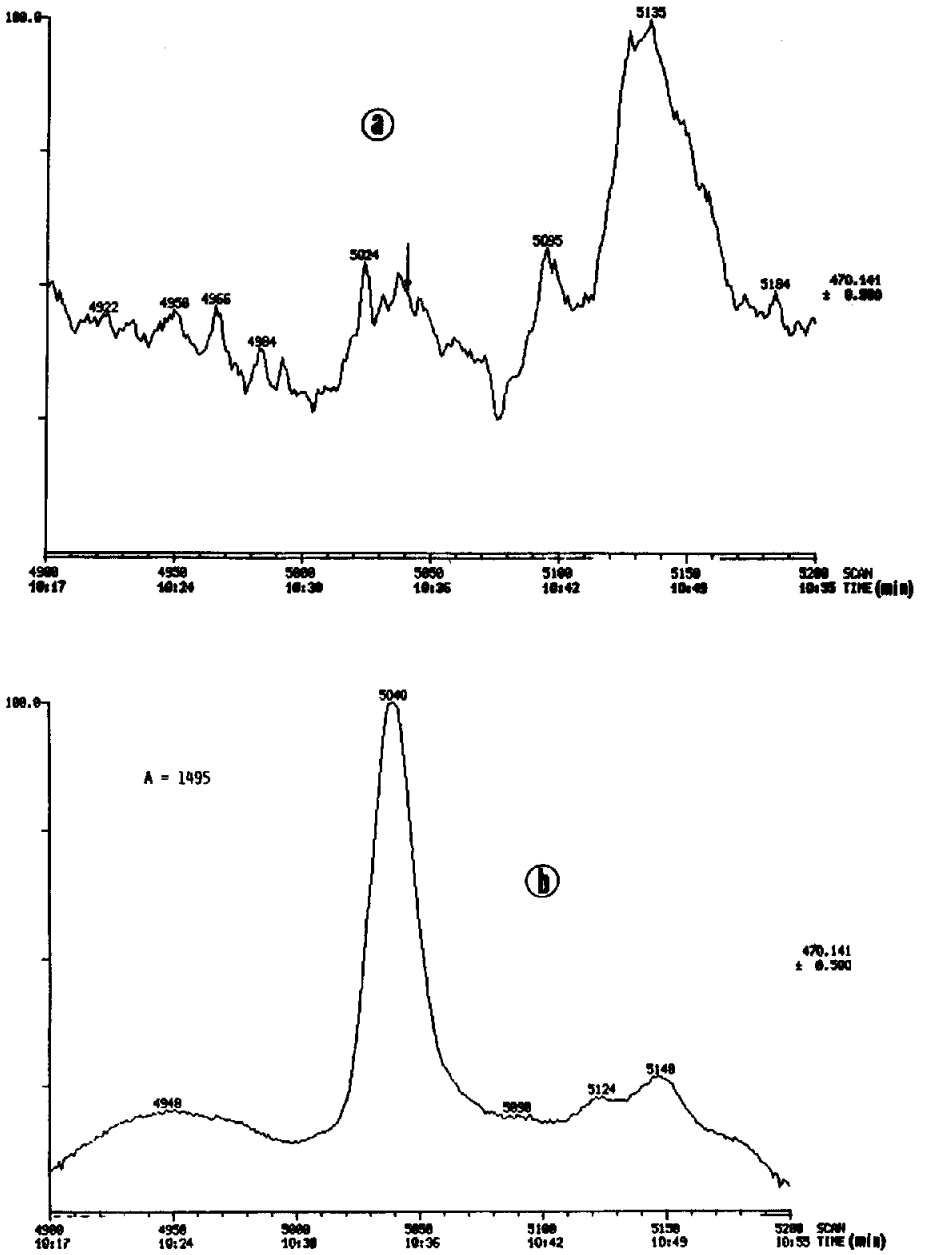

Fig. 2. (a) SIM chromatogram of *m/z* 470 of urine, treated to eliminate endogenous salsolinol, showing the absence of a peak at the retention time of salsolinol (arrow); (b) SIM chromatogram of *m/z* 470 of urine made free of endogenous salsolinol and spiked with 0.55 pmol/ml salsolinol; for comparison, area (*A*) of the peak of *m/z* 474 was 45026.

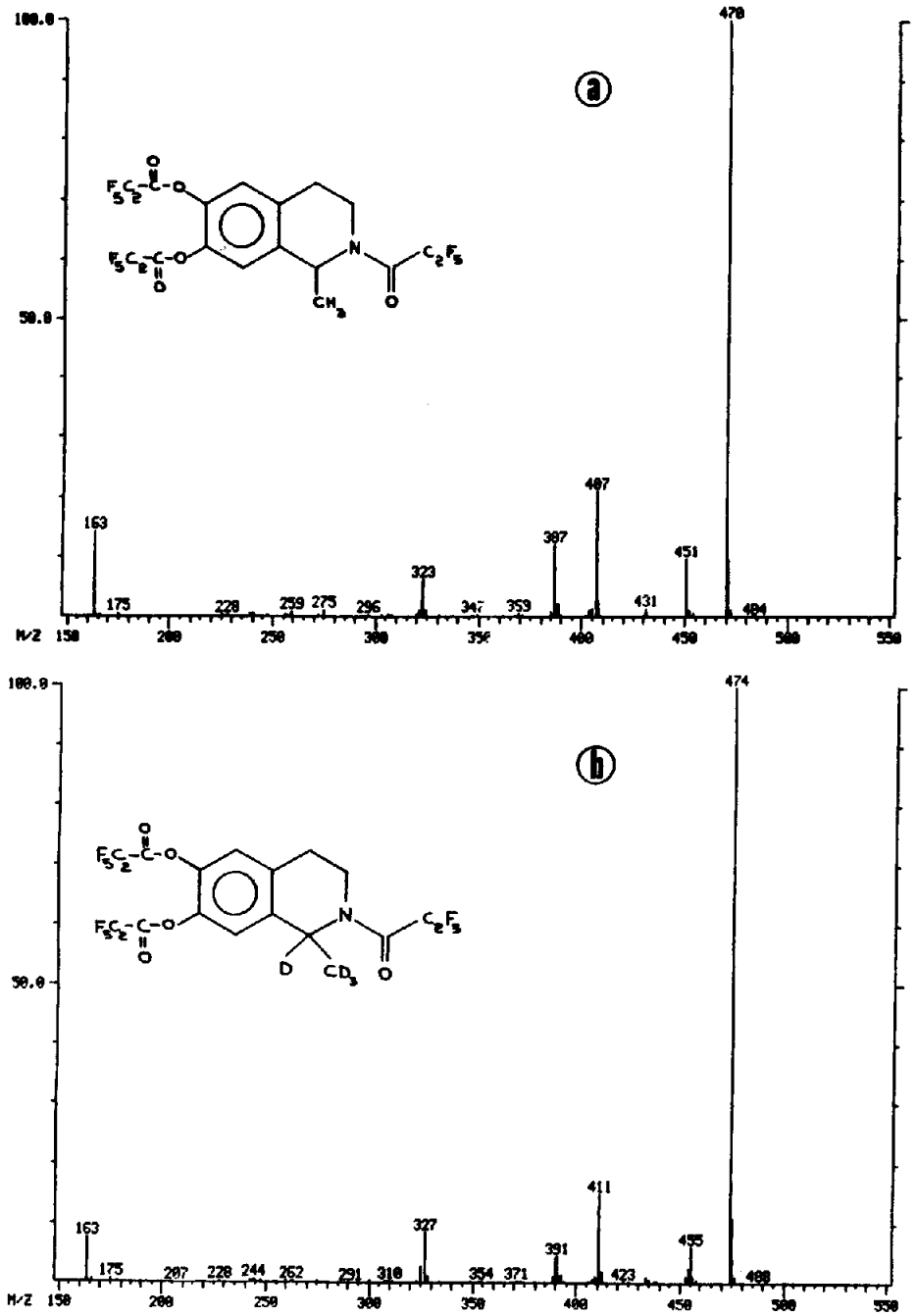

Fig. 3. EC-NICI mass spectra of PFPA derivatives of (a) salsolinol and (b) [$^2H_4$]salsolinol (I).

Analytical data were obtained in the SIM mode. The ions selected were *m/z* 470 (salsolinol) and 474 ([$^2H_4$]salsolinol) ([M-147]$^-$). The scan-rate was 0.05 s for each mass interval.

Calibration curves

The calibration curves were established using water-diluted urine samples made free from endogenous salsolinol according to the following procedure: urine kept at pH 12 for 12 h was then diluted with water (1:4), brought to pH 8 and filtered through a PBA cartridge. No salsolinol was detectable in the eluate (Fig. 2a). Samples of eluate were spiked with amounts of salsolinol ranging from 0.55 to 144 pmol/ml. Each sample contained 17 pmol/ml I as internal standard and was submitted to the whole procedure of extraction and derivatization. Standard curves were generated by least-squares linear regression analysis.

RESULTS

The EC-NICI mass spectra of the pentafluoropropionyl derivatives of salsolinol and [$^2H_4$]salsolinol (I) are shown in Fig. 3. The base peaks (*m/z* 470 and 474) are not the quasi-molecular anions, but fragments corresponding to [M − COC_2F_5]$^-$. Note that no isotopic contamination of deuterium-labelled salsolinol was found in salsolinol, and *vice versa*.

The linearity was evaluated from six different calibration curves carried out in the whole range of measurement on six different days. The mean calibration curve was defined by the equation $y = 0.964x + 0.048$ [slope coefficient of variation (C.V.) = 8.7%] (correlation coefficient $r = 0.999$); the y-intercept values, when submitted to the Student's t-test, were not significant different from zero ($p > 0.05$).

TABLE I

PRECISION OF THE METHOD (INTRA-DAY ASSAY)

Amount (n = 3) (pmol/ml)	Response factor[a] (mean ± S.D.)		
	First day	Second day	Third day
144.13	0.99 ± 0.05	0.97 ± 0.01	0.93 ± 0.03
5.76	0.99 ± 0.03	1.02 ± 0.05	0.99 ± 0.03
0.57	1.01 ± 0.04	1.08 ± 0.02	0.99 ± 0.03
	1.00 ± 0.03	1.03 ± 0.06	0.97 ± 0.04
C.V. (%)	3.1	5.4	4.2

[a] The response factor is calculated from (peak area salsolinol/peak area internal standard) × (amount internal standard/amount salsolinol).

TABLE II

PRECISION OF THE METHOD (INTER-DAY ASSAY)

Response factor (mean ± S.D.)	C.V. (%)
1.00 ± 0.03	3.1
1.03 ± 0.06	5.4
0.97 ± 0.04	4.2
1.0 ± 0.05	4.8

The C.V. of the intra- and inter-day precision were determined using three different concentrations analysed in triplicate on the same day and on three different days, and was found to be 3.1–5.4% and 4.8%, perspectively (Tables I and II). The accuracy over the whole range of interest, calculated from three different concentrations (0.87, 5.7 and 115.3 pmol/ml) analysed in triplicate on three different days and reported as the mean percentage ratio of the found/added amounts, was 98.1% (C.V. = 8.1%). The recovery of the method determined using a concentration of 0.57 pmol/ml was found to be 70–75%. The minimum detectable amount was assumed to be the lowest validated point of the standard curve, *i.e.* 0.55 pmol/ml.

Concentrations of free salsolinol in the 24-h urine from the six healthy volunteers are given in Table III. A typical SIM chromatogram of urine from subject M.C. is shown in Fig. 4.

TABLE III

URINE CONCENTRATIONS AND DAILY OUTPUT OF FREE SALSOLINOL IN SIX HEALTHY SUBJECTS

Subject	Concentration (pmol/ml)	24-h Excretion (nmol)
A.D.	1.7	1.8
F.D.	4.4	8.0
M.C.	1.0	1.7
M.P.	2.2	4.1
P.P.	6.1	4.4
P.S.	3.6	4.9

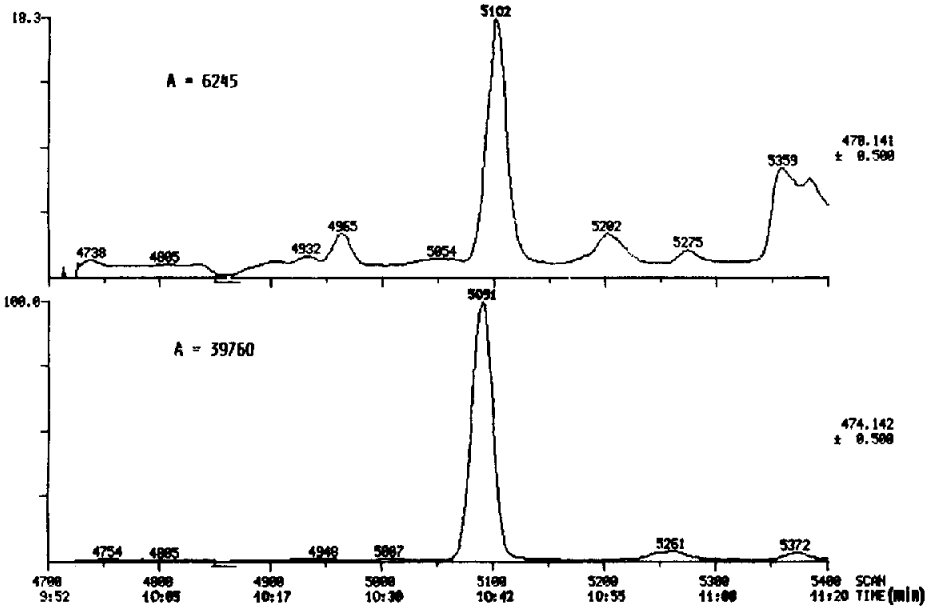

Fig. 4. SIM chromatogram of *m/z* 470 and 474 (internal standard) of a urine sample of subject M.C. (*A* area of the peak).

DISCUSSION

The enhanced sensitivity inherent in the EC-NICI technique was realized by using the PFPA derivative of salsolinol to produce stable anions. The ionization efficiency obtained was *ca.* fifty-fold higher than that obtained using positive-ion CI and electron-impact ionization with the same PFPA derivative.

The use of capillary columns in GC has long been known to lead to improved resolution and sensitivity. However, a distinct disadvantage of capillary columns is their limited sample capacity. Urine concentrations of salsolinol are usually quite low, so that the amount of salsolinol injected into the gas chromatograph should not exceed the column capacity. However, various other polyhydroxy derivatives are also present in urine, often in concentrations greater than that of salsolinol. In fact, the use of narrow-bore and wide-bore capillary columns for the determination of urine concentrations of salsolinol was found to give non-reproducible results, the column sensitivity declining rapidly on repeated injections. A good solution was achieved by using megabore columns, which have the high capacity of packed columns with higher resolution and reproducibility.

When urine concentrations of (*R*)- and (*S*)-salsolinol were determined in parkinsonian patients before and after administration of L-dopa [16], using the HPLC–ED method set up by Pianezzola *et al.* after formation of diastereoisomeric derivatives [17], concentrations of either salsolinol enantiomer were found

to be lower than the limit of detection (14 pmol/ml) in the absence of L-dopa. Using the same methodology, the urine levels of salsolinol enantiomers were sometimes found to be lower than 14 pmol/ml in healthy volunteers [15] and, surprisingly, remained under the limit of detection in one subject even after administration of L-dopa. Though not permitting the determination of salsolinol enantiomers, the new EC-NICI method described in this paper considerably improves the possibility of measuring very low urine concentrations of total ($R + S$) salsolinol. This should be of practical use if determination of urine concentrations of salsolinol might contribute to the early detection of Parkinson's disease, as urine concentrations of free salsolinol were found to vary from *ca.* 1 to 6 pmol/ml in the six volunteers of this study and should be lower in parkinsonian patients.

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