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IDENTIFICATION AND QUANTIFICATION OF 1-CARBOXYSALSOLINOL AND SALSOLINOL IN BIOLOGICAL SAMPLES BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

BIRGITTA SJÖQUIST* and CHARLOTTA LJUNGQUIST

Department of Alcohol and Drug Addiction Research, Karolinska Institutet, S-104 01 Stockholm (Sweden)

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SUMMARY

1-Carboxysalsolinol was found to be present in rat striatum, human urine and caudate nucleus of post mortem human brain, according to capillary column gas chromatographic retention times and selected ion monitoring of the hexafluoropropionyl ester pentafluoropropyl derivative. Simultaneous quantification of 1-carboxysalsolinol and salsolinol was performed in biological samples using deuterium labelled internal standards. In human urine, the precision of the method was ± 7.1% (coefficient of variation, n = 25) for 1-carboxysalsolinol at 15 pmol/ml and ± 8.5% for salsolinol at 10 pmol/ml. According to enzymatic hydrolysis, 68% of 1-carboxysalsolinol was found as conjugates in urine, and the corresponding figure for salsolinol was 92%. In human caudate nucleus, the amounts of 1-carboxysalsolinol were found to be significantly greater in brains from alcoholics, who at autopsy had ethanol present in the blood, whereas alcoholics without blood ethanol levels at autopsy had significantly lower concentrations of salsolinol.

INTRODUCTION

Acetaldehyde can condense with dopamine and form 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, which is called salsolinol [1]. Salsolinol has been found in urine [2- 4] from both alcoholics and control individuals, as well as in cerebrospinal fluid [3, 4]. It has also been found in rat brain [5—7] and human brain tissue [8]. The original hypothesis was that this tetrahydroisoquinoline alkaloid could be formed after ethanol administration and that it could be responsible for the addiction properties of ethanol [9, 10]. However, salsolinol has been found also without prior ethanol exposure. Acute ethanol administration to healthy human volunteers has not

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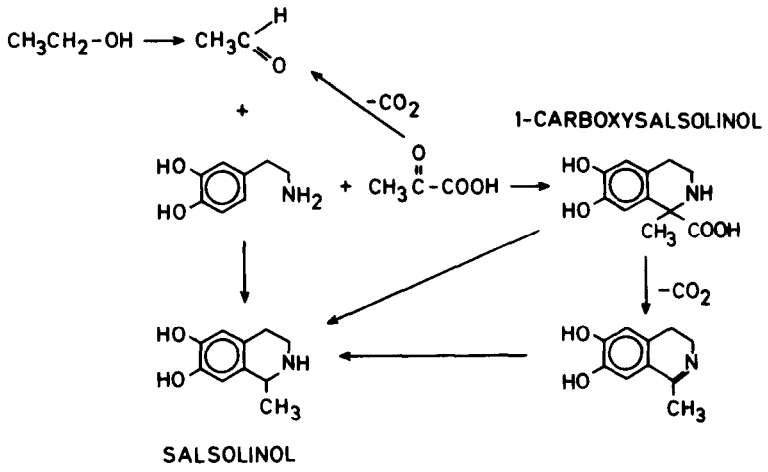

Fig. 1. The condensation reaction between pyruvate and dopamine, and different possibilities for salsolinol formation.

resulted in elevated salsolinol levels [4]. These data are in agreement with recent reports that in healthy Caucasians the blood contains at most only small amounts of acetaldehyde (less than 1 μM) after moderate alcohol consumption [11]. It is only after chronic exposure to ethanol that salsolinol has been found to increase [2, 3, 6–8]. Even then there has been an inverse correlation between blood ethanol levels and brain salsolinol concentration [6, 8]. These observations make unlikely the original hypothesis that salsolinol would simply be formed from dopamine and acetaldehyde, derived from the ethanol metabolism. Furthermore, many alcoholic beverages have been found to contain salsolinol [12]. That makes it difficult to interpret elevated levels in urine from alcoholics.

In the light of these experiences we suggest that salsolinol formation is linked to an effect of ethanol on the energy metabolism. Pyruvate is a key substance in carbohydrate metabolism. Pyruvate is also a low-molecular-weight α-keto acid that can condense with dopamine. In the peyote cactus, ^{14}C-labelled pyruvate has been shown to condense with demethylmescaline to form the corresponding 1-carboxytrihydroisoquinoline, and the latter alkaloid was further decarboxylated to anhalonidine [13]. If dopamine condenses with pyruvate in a Pictet–Spengler reaction the product would be 1-carboxysalsolinol [14]. This alkaloid may be the precursor of salsolinol, or pyruvate could form acetaldehyde that can directly condense with dopamine to form salsolinol (see Fig. 1). The purpose of this study was to investigate if 1-carboxysalsolinol is present in biological samples and, when it was detected, to develop a sensitive and selective method for the simultaneous measuremement of salsolinol and 1-carboxysalsolinol in biological samples.

EXPERIMENTAL

Materials

Salsolinol was synthesized as described by Schöpf and Bayerle [1], and 1-carboxysalsolinol was obtained from pyruvate and dopamine as described

below. Both compounds were labelled with deuterium atoms in positions 5 and 8 through an acid-exchange reaction. Propionic anhydride (PFPA) was obtained from Reagenta (Uppsala, Sweden), hexafluoroisopropanol (HFIP) from Fluka (Buchs, Switzerland) and sulphatase (H-1) from Sigma (St. Louis, U.S.A.).

Preparation of 1-methyl-1-carboxy-6,7-dihydroxy-2,3,4-trihydroisoquinoline (1-carboxysalsolinol)

Sodium pyruvate (1.11 mmol) was dissolved in distilled water (1.2 ml). Dopamine hydrochloride (0.53 mmol) was added. After 24 h the white precipitate was filtered off and washed with distilled water.

Preparation of 1-methyl-1-carboxy-5,8-dideutero-6,7-dihydroxy-2,3,4-trihydroisoquinoline

1-Carboxysalsolinol (0.50 mmol) was dissolved in 10% ^{2}HCl by ultrasonification. The solution was protected from light and heated to 130—140°C overnight. The solvent was evaporated and the crude product was redissolved in a small amount of methanol. This solution was added slowly to chilled diethyl ether until white crystals precipitated.

Conditions for enzymatic hydrolysis of conjugates

Urine was pooled from a 24-h collection period from five healthy human males (38—47 years old). The urine samples (2 ml) were incubated at 37°C with 10 or 20 mg of sulphatase at pH 4—7 for 0.5—20 h, then analysed as described below.

Analysis of 1-carboxysalsolinol and salsolinol

In urine. To human urine (2 ml) was added a water solution (1 ml) containing 10 mg of semicarbazide hydrochloride, 2 mg of $NaHSO_3$ and 30 mg of ethylenediamine tetraacetate (EDTA) and the internal standards [$^2H_2$]-1-carboxysalsolinol (0.1 nmol for free and 0.25 nmol for analysis of total 1-carboxysalsolinol) and [$^2H_2$]salsolinol (0.1 nmol or 0.5 nmol, respectively). The samples were incubated at pH 6.5 for 16—20 h at 37°C with 10 mg of sulphatase. Following incubation of the samples the pH was adjusted to 8.55—8.65 with 1 *M* hydroxymethylaminomethane (Tris) buffer and 200 mg of Al_2O_3 were added. After 15 min of shaking the supernatant was sucked off and the Al_2O_3 was washed with distilled water (3 × 1 ml). The catechols were eluted with 2 × 200 µl of 0.5 *M* formic acid. The samples were lyophilized or evaporated under vacuum at 40°C. The unconjugated compounds were determined as described above but without incubation with sulphatase.

In brain tissue. Human caudate nucleus was obtained from ten alcoholics and five control cases. At autopsy, six of the alcoholics had ethanol in the blood. Tissue samples were weighed and transferred to high-speed centrifugation tubes. The samples were homogenized with an Ultra Turrax in a solution containing semicarbazide hydrochloride (10 g/l), $NaHSO_3$ (0.2%), and EDTA (0.1 nmol). After centrifugation at 20 000 *g*, the supernatant was transferred to a test tube containing Al_2O_3 (50 mg). The samples were then processed as the urine samples.

Derivatization

The dry samples were dissolved in 20 μl of HFIP and 80 μl of PFPA and heated for 1 h at 60°C. After evaporation to dryness under a stream of nitrogen the samples were redissolved in ethyl acetate in PFPA (1:1) and kept at 60°C for 10 min. The reagent was evaporated and the samples were finally dissolved in 30 μl of ethyl acetate before analysis by gas chromatography—mass spectrometry (GC—MS).

Instrumental conditions

An LKB 2091 and a Finnigan 4021 combined gas chromatograph—mass spectrometer were used. The LKB instrument was equipped with a multiple-ion detector and a 1% OV-17 column, length 2.5 m. The flow-rate of the carrier gas (helium) was 15—20 ml/min. Temperatures were: column 190°C; flash heater, 230°C; ion source, 270°C. For selected-ion monitoring the energy of the electrons were 22.5 eV. The Finnigan instrument was equipped with a fused-silica column, Se-54, length 25 m, and an Incos data system. The energy of the electrons was 70 eV. The ions selected to run were *m/z* 617/619 (M^+) and 602/604 ($M^+ - 15$) for salsolinol, and *m/z* 350/352 and 616/618 ($M^+ - 195$) for 1-carboxysalsolinol.

Precision and recovery

Urine was pooled from five healthy male volunteers (37—42 years old) after a 24-h collection period. Aliquots of 2 ml were analysed without and with enzymatic hydrolysis.

Analysis of individual urine samples

Urine samples were collected from ten healthy male individuals (25—44 years old) during 10 h. These samples were analysed according to the described method after enzymatic hydrolysis.

Control of artifact formation

Dopamine labelled with four deuterium atoms in the side-chain was added to urine samples (2 ml), 2 nmol to samples that were not hydrolysed and 5 nmol to samples that were hydrolysed. If dopamine condensed with acetaldehyde or pyruvate during the work-up procedure, salsolinol and 1-carboxysalsolinol containing four deuterium atoms would be formed.

RESULTS

The yield of 1-carboxysalsolinol was 43%. After derivatization with HFIP and PFPA it gave a single GC peak on the Se-54 capillary column, and a mass spectrum run in the electron impact mode as shown in Fig. 2. The base peak in the spectrum was *m/z* 616, i.e. the molecular ion minus $COOCH(CF_3)_2$ ($M^+ - 195$). The corresponding mass spectrum for the deuterium-labelled 1-carboxysalsolinol is also shown in Fig. 2. Fragment *m/z* 350/352 was used in combination with *m/z* 616/618 to check identity.

On the 1% OV-17 column, the hexafluoroisopropyl ester pentafluoropropionyl derivative of 1-carboxysalsolinol had a retention time of 2 min at

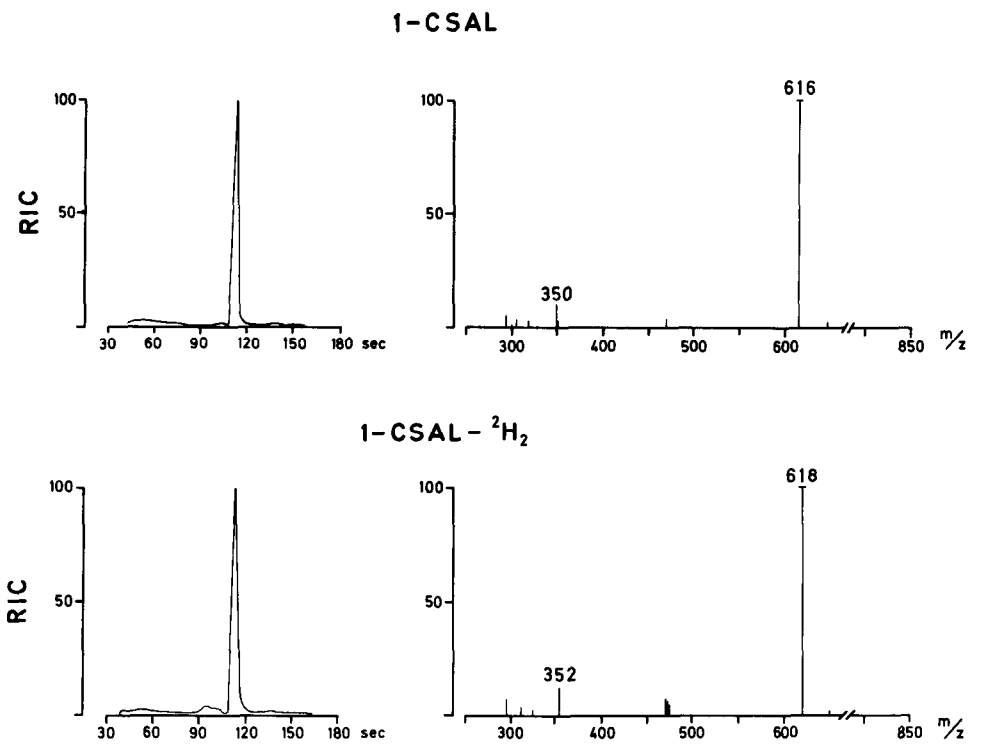

Fig. 2. The gas chromatograms and mass spectra of the hexafluoroisopropyl ester pentafluoropropionyl derivatives of 1-carboxysalsolinol (1-CSAL) and 5,8-dideutero-1-carboxysalsolinol (1-CSAL-$^2H_2$).

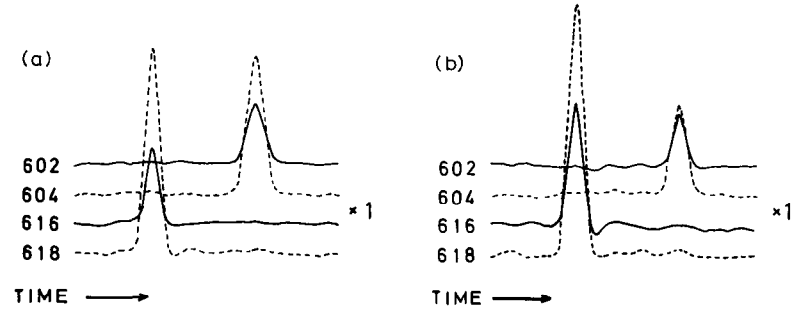

Fig. 3. Selected-ion monitoring of *m/z* 616/618 from the hexafluoroisopropyl ester pentafluoropropionyl derivative of 1-carboxysalsolinol and *m/z* 602/604 from the pentafluoropropionyl derivative of salsolinol in urine samples (a) before and (b) after enzymatic hydrolysis. Broken lines represent the deuterium-labelled internal standards and the solid lines the endogenous found compounds.

190°C and a flow-rate of 20 ml/min. The pentafluoropropionyl derivative of salsolinol had a retention time of 2.5 min under the same conditions. Selected-ion monitoring of urine samples is shown in Fig. 3.

In the hydrolysis experiment, it was found that the optimal conditions for cleaving salsolinol conjugates in urine were incubation at pH 6.5–7 for 15–20 h, whereas 1-carboxysalsolinol conjugates were optimally hydrolysed after only

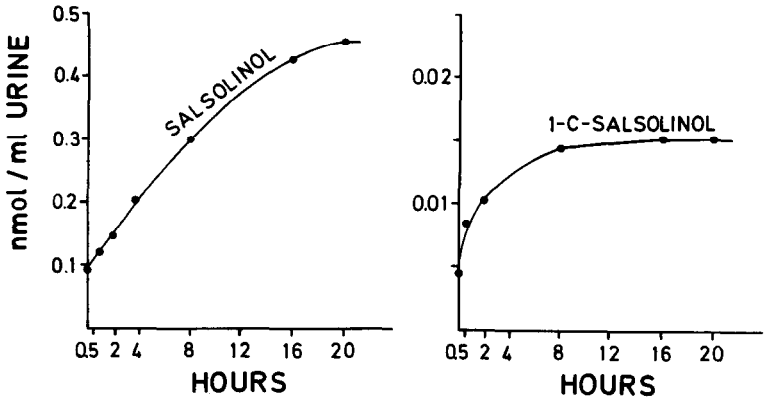

Fig. 4. The influence of time on the enzymatic hydrolysis of conjugates with sulphatase containing β-glucuronidase at 37°C and pH 6.5. The urine samples used were from a different pool than the urine used in the precision and recovery experiment.

TABLE I

CONCENTRATIONS OF 1-CARBOXYSALSOLINOL AND SALSOLINOL IN HUMAN BRAIN TISSUE

Mean ± S.E.M., according to Students *t*-test.

	Human caudate nucleus			
	1-Carboxysalsolinol (pmol/g)	*n*	Salsolinol (pmol/g)	*n*
Control	5.9 ± 2.4	5	93 ± 31	5
Alcoholics with blood ethanol	25 ± 8.1*	6	60 ± 5.5	6
Alcoholics without blood ethanol	8.5 ± 2.0	4	30 ± 8.5**	4

*$p < 0.05$ compared with control.
**$p < 0.01$ compared with alcoholics with blood ethanol.

8 h (see Fig. 4). An adequate amount of enzyme was 10 mg of sulphatase per 2 ml urine, since 20 mg did not increase the recovery. This hydrolysis experiment was performed on a urine pool different from the urine pool used to study the precision.

In the urine pool analysed in the precision study, free 1-carboxysalsolinol was found in a concentration of 15.4 ± 1.1 pmol/ml (mean ± S.D.), which rose to 47.9 ± 6.1 pmol/ml after incubation with sulphatase. The corresponding values for salsolinol were 11.2 ± 0.95 pmol/ml and 139 ± 17.8 pmol/ml, respectively. When the same samples were subjected to GC—MS on two different occasions, the instrumental variation for free 1-carboxysalsolinol was 5.7%, rising to 10% after enzymatic hydrolysis. The corresponding variations for salsolinol were 7.8% and 8.5%, respectively.

The urine samples analysed from ten different individuals gave a mean value for 1-carboxysalsolinol of 16.5 ± 2.24 pmol/ml (range 7.9—31.0 pmol/ml). The corresponding value for salsolinol was 199 ± 48.6 pmol/ml (range 61–445 pmol/ml).

The results of the analysis of 1-carboxysalsolinol and salsolinol in human caudate nucleus are shown in Table I.

In the experiment where deuterium-labelled dopamine was added there were no signs of artifact formation of either salsolinol or 1-carboxysalsolinol.

DISCUSSION

Pyruvate reacted readily with dopamine to form 1-carboxysalsolinol. The condensation of α-keto acids with hydroxyphenylethylamines was thoroughly investigated by Hahn and Stiehl in 1936 [14]. They also found that these 1-carboxytrihydroisoquinoline alkaloids could spontaneously decarboxylate under physiological conditions. It was shown by Kapadia et al. in 1970 that ^{14}C-labelled pyruvate injected into peyote cactus was incorporated in the tetrahydroisoquinoline alkaloid anhalonidine [13].

In this study no attempts were made to optimize the recovery of the synthesis. According to GC—MS of the hexafluoroisopropyl ester pentafluoropropionyl derivative of 1-carboxysalsolinol the compound was pure. This product was used to synthesize the deuterium-labelled internal standard. In the deuterium exchange reaction the incorporation of two deuterium atoms was optimal after 20 h. A longer reaction time lead to incorporation of two to five deuterium atoms. For derivatization of the carboxylic acid group pentafluoropropanol was also tried, but the hexafluoroisopropyl derivative gave a better separation from the salsolinol derivative.

The selectivity of the method was tested by analysis of *m/z* 350/352 in addition to *m/z* 616/618 for the derivative of 1-carboxysalsolinol, and of *m/z* 602/604 and *m/z* 617/619 (M^+) for salsolinol. The same results were obtained at both sets of ions for both 1-carboxysalsolinol and salsolinol. The most intense ions were used in the quantitative analysis. The detection limit of the method was 1 pmol per millilitre of urine, or 1 pmol per sample. 1-Carboxysalsolinol was found in urine for the first time to our knowledge; most was excreted as conjugates (68%). The amount of free 1-carboxysalsolinol was in the same range as the amount of salsolinol found, while the total amount of 1-carboxysalsolinol was lower than the total amount of salsolinol. In the rat it has been shown that 1-carboxysalsolinol can be methylated. After an intracerebral ventricular injection, 12% of the recovered tetrahydroisoquinoline compounds was found as 7-O-methylated 1-carboxysalsolinol [15]. The proportion of O-methylated 1-carboxysalsolinol in human urine is thus far unknown.

1-Carboxysalsolinol was also found in human caudate nucleus. The levels of 1-carboxysalsolinol in brain tissue were somewhat lower than the salsolinol concentrations. In the brain tissue of alcoholics with ethanol in the blood at autopsy, 1-carboxysalsolinol was found in higher concentrations than in control cases, whereas alcoholics without blood ethanol levels at autopsy did not differ from control cases. This might indicate that the pyruvate dehydrogenase complex is inhibited by acetaldehyde derived from ethanol, thus the decarboxylation of pyruvate would be inhibited and the condensation of dopamine with pyruvate would be favoured. It has been shown in vitro that acetaldehyde can inhibit the pyruvate dehydrogenase complexes isolated from ox brain and ox kidney [16] and from rat liver mitochondria [17]. The

decreased level of salsolinol in caudate nucleus from alcoholics with ethanol in the blood has been observed earlier in a more extensive study [8].

In conclusion, a sensitive and selective method has been developed for the simultaneous quantitative measurement of 1-carboxysalsolinol and salsolinol in body tissues and fluids. The discovery of these dopamine-related tetrahydroisoquinoline alkaloids in human urine and brain tissue is of potential interest in alcohol research, and might reflect an ethanol interaction with the energy metabolism. These alkaloids can also turn out to be neurotoxic by mechanisms similar to those for 6-hydroxydopamine.

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