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Short communication

Simultaneous structure–activity determination of disulfiram photolysis products by on-line continuous-flow liquid secondary ion mass spectrometry and enzyme inhibition assay

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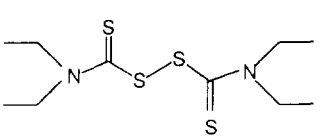
Abstract

Disulfiram (DSF) is used in the treatment of recovering alcoholics and exerts its effect by inhibiting the enzyme aldehyde dehydrogenase (ALDH). We analyzed a mixture of products derived photochemically from DSF with on-line microbore HPLC–continuous-flow liquid secondary ion mass spectrometry (HPLC–CF-LSI-MS). By utilizing the post-HPLC column split of solvent flow, a small proportion (ca. 5%) was sent directly into the mass spectrometer, and the remainder was collected. Simultaneous MS analysis and enzyme inhibition studies on ALDH were then possible. Furthermore, using HPLC–CF-LSI-MS–MS, we were able to structurally characterize an interesting sulfine compound that inhibited ALDH.

1. Introduction

The dithiocarbamate disulfiram (DSF) is the only commercially available therapeutic agent (trade name Antabuse) used in the treatment of recovering alcoholics [1]. The ethanol-sensitizing effect of DSF is due to its irreversible inhibition of the enzyme aldehyde dehydrogenase (ALDH). Inhibition of ALDH leads to elevated levels of acetaldehyde in the bloodstream, which results in nausea, vomiting, flushing and tachycardia in individuals after ingestion of ethanol [1]. We have demonstrated recently that the dithiocarbamate, sulfiram, is unstable in solution [2] and is readily photoconverted to products, including DSF, that inhibit ALDH [3]. We undertook this investigation to determine if DSF, which is structurally similar to sulfiram, is also photoconverted to products that inhibit ALDH.



(DSF)

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Continuous-flow liquid secondary ion mass spectrometry (CF-LSI-MS) is a useful and sensitive technique for the analysis of mixtures when coupled to microbore HPLC [4]. However, it is necessary to split the HPLC flow in order to accommodate the vacuum constraints of the mass spectrometer. Typically the sample splitting allows only a small portion of the analyte (ca. 5%) into the MS system while sending a majority to waste (ca. 95%). Traditionally, CF-LSI-MS has been thought of as "limited" by such low flow-rates (ca. 1–3 μl/min into MS), but we have found that the splitting of sample can be useful for another application. By recovering the HPLC eluent of a mixture derived from photochemically treated DSF destined for "waste", and subjecting fractions to bioassay, it was possible to ascertain the inhibitory activity of specific compounds against ALDH. Combining this information with the structural analysis provided by HPLC–MS and HPLC–tandem mass spectrometry (HPLC–MS–MS), it was possible to characterize on-line the structure–activity relationship of DSF and photolytically produced products.

2. Experimental

2.1. Reagents and materials

DSF was purchased from Sigma (St. Louis, MO, USA) and recrystallized twice in ethanol before use (m.p. 71–72°C). Aldehyde dehydrogenase [aldehyde: NAD(P) oxidoreductase, EC 1.2.1.5; specific activity: 51 units/mg enzyme protein], isolated from *Saccharomyces cerevisiae*, NAD (grade 1 free acid, 100%), Tris · HCl and Tris base were purchased from Boehringer Mannheim (Mannheim, Germany). Acetaldehyde and KCl were obtained from Aldrich (St. Louis, MO, USA). Acetonitrile and ethanol (redistilled before use) were obtained from Baxter (McGaw Park, IL, USA).

2.2. Photolysis of DSF

DSF was dissolved in acetonitrile under subdued light to afford an 85 m*M* solution and subsequently subjected to photolysis. Dark control solutions of DSF in glass vials with PTFE-lined screw caps were wrapped in foil and stored at room temperature. Other solutions of DSF were irradiated at 254 nm for 4 h at 31°C in a Rayonet Photochemical Reactor (Model RMR-600; Southern New England Ultraviolet, Branford, CT, USA). Light intensity at the center of the photochemical reactor was 12.8 μW/cm^2.

2.3. HPLC–CF-LSI-MS

HPLC separations were performed on an ultrafast microprotein analyzer (UMA) system (Michrome Bioresources, Pleasanton, CA, USA). Samples of the DSF photolysis reaction mixture (2-μl aliquots in acetonitrile) were injected directly into the HPLC system and analytes were separated on a Michrome UMA microbore column (150 mm × 1 mm I.D.) with Reliasil C_{18} (100 Å, 3 μm) packing material. Separations were achieved using a mobile phase of acetonitrile–water–glycerol (50:48:2, v/v) delivered isocratically for 15 min followed by a 10-min linear gradient to acetonitrile–water–glycerol (80:18:2, v/v) at a flow-rate of 59 μl/min. Absorbance at 225 nm was continuously monitored. A post-column split of 1:20 allowed ca. 3 μl/min of eluent to enter into the mass spectrometer and the remainder was sent to "waste" (56 μl/min) and recovered and subjected to bioassay for ALDH inhibitory activity as shown schematically in Fig. 1. Prior to bioassay, the recovered material was lyophilized to remove excess solvent.

All HPLC–CF-LSI-MS and HPLC–CF-LSI-MS–MS experiments were performed on a Finnigan MAT 95Q mass spectrometer (Bremen, Germany) of BE Q_1Q_2 configuration, where B is the magnet, E is the electrostatic analyzer, Q_1 is a radio frequency (rf)-only octapole collision cell and Q_2 is a mass filter quadrupole. Samples were ionized by CF-LSI-MS in positive mode using cesium as a source of primary ions at an ion voltage of 20 kV (Cs^+ ions). The CF-LSI-MS probe consisted of a silica capillary (50 μm I.D. × 200 μm O.D.) connected directly to the HPLC. The ion source was maintained at 60°C

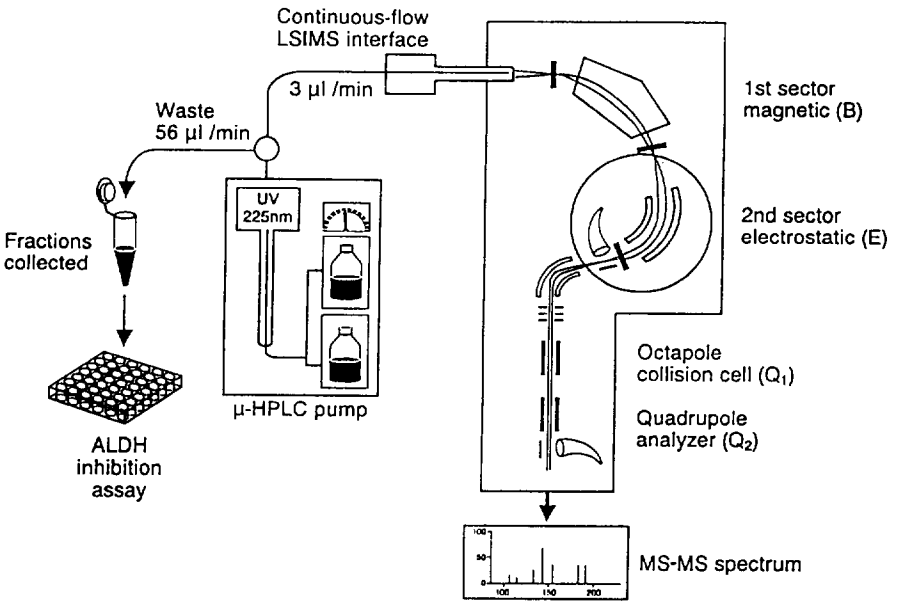

Fig. 1. Schematic of the instrumentation used in the simultaneous structure–activity determination of disulfiram photolysis products employing a CF-LSI-MS interface connected to a MAT 95Q hybrid mass spectrometer.

with an operating resolution of ca. 1200, scanning over a mass range of 50–300 u at 3 s/decade.

2.4. Tandem mass spectrometry

Molecular ions (also referred to as precursor ions) of standards and photolysis products of DSF were selected with a resolution of ca. 1000 using BE (MS1) and subjected to collision-induced dissociation (CID) in the octapole collision cell Q1 using argon as the collision gas. Collision energies of ca. 20–ca. 30 eV with an argon gas pressure of ca. $6 \cdot 10^{-6}$ mbar were used to induce CID of precursor ions. The product (fragment) ions were mass analyzed in Q_2 (MS2) and product ion spectra acquired by scanning Q2 over the mass range of 40 to 350 with 10 to 20 scans acquired and averaged to afford a composite product ion spectrum.

2.5. ALDH assay

The activity of yeast ALDH was assayed with a microplate reader (Molecular Devices, Menlo Park, CA, USA) at 22°C by following the formation of NADH spectrophotometrically at 340 nm. All reagents were prepared in 50 m*M* sodium pyrophosphate buffer, pH 8.8. The assay mixture in a final volume of 200 µl contained the following: sodium pyrophosphate (pH 8.8):1.5 m*M* NAD; 0.03 units yeast ALDH; 2.4 m*M* acetaldehyde; and DSF or DSF photolysis products (collected from HPLC) in 1.75 µl of ethanol (0.88%, v/v, final concentration of ethanol). An equal volume of ethanol or mobile phase was added to control assays without inhibitor. The order of addition of reagents was sodium pyrophosphate, yeast ALDH, NAD and inhibitor. The solutions were mixed by gently tapping the microtiter plate, and the substrate acetaldehyde (2.4 m*M* final concentration) was added to start the reaction. The solutions were again mixed and the initial rates obtained by following the absorbance at 340 nm for 3 min at 22°C.

3. Results and discussion

Fractions from the HPLC "waste" eluent containing individual components were assayed for inhibition of ALDH. Substantial inhibition

was observed for a number of compounds that eluted in the solvent front, as well as late eluting components at retention times of ca. 27, 29.5 and 39.5 min (see Fig. 2). However, complete (100%) inhibition of ALDH was detected for individual compounds at retention times of ca. 7.5, 9.5 and 19.5 min (Fig. 2). The compound eluting at ca. 13 min, based on relative retention time compared to authentic standard, was determined to be unphotolyzed and/or regenerated DSF, and this was subsequently confirmed by MS analysis. However, due to the high concentration of DSF in this fraction, precipitation occurred which adversely affected the enzyme inhibition assay. We have determined previously that the IC_{50} of DSF on yeast ALDH is ca. 2 μM [3].

Concomitant HPLC–CF-LSI-MS analysis of the photolysis mixture revealed molecular ions at $MH^+ = 281$ [retention time (t_R) 7.5 min]; $MH^+ = 313$ ($t_R = 9.5$ min); $MH^+ = 297$ ($t_R = 13$ min) corresponding to unphotolyzed DSF; and $MH^+ = 212$ ($t_R = 27$ min) as shown in Fig. 3. No clearly discernible molecular ions were detected for solvent front components ($t_R = 0$–5 min) nor for late-eluting active components ($t_R = 19.5$, 29.5 and 39.5 min) (Fig. 3).

In order to determine structures of the components at m/z 281, 313, 297 and 212, the precursor ions were subjected to MS–MS via collision induced dissociation with the target gas argon contained in an rf-only octapole collision cell. The details of this work will be reported elsewhere. However, of major interest was the

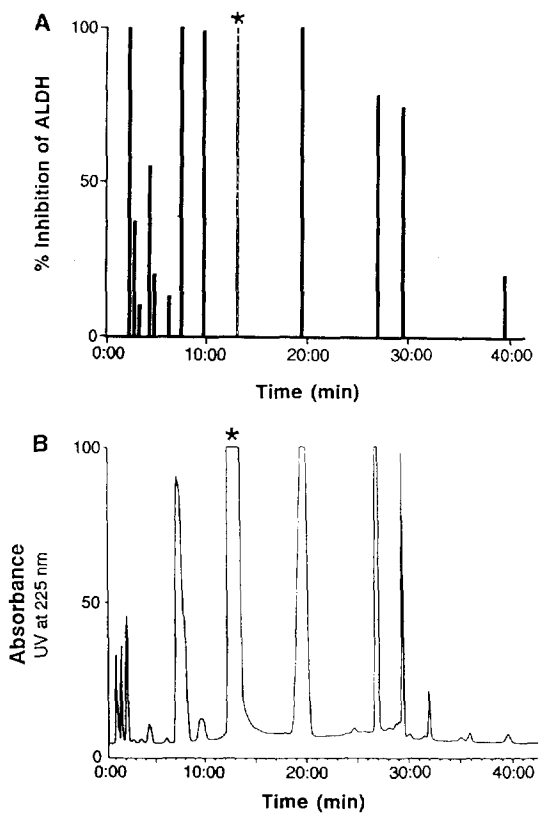

Fig. 2. Inhibitory activity of DSF photolysis products against the enzyme ALDH after reversed-phase HPLC separation. (A) Enzyme inhibition (%) of photolysis products of ALDH; (B) HPLC chromatogram with UV detection of photolysis products of disulfiram (at 225 nm). (*) This peak corresponds to DSF which, because of its high concentration, precipitated and interfered with the enzyme inhibition assay.

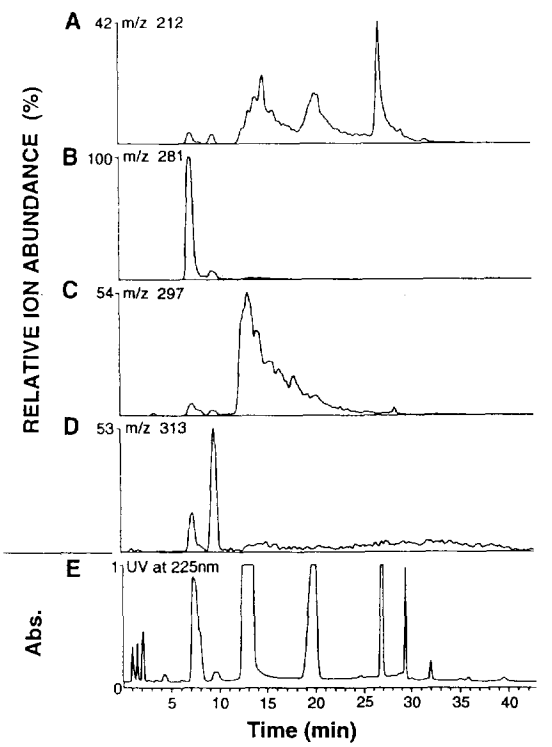

Fig. 3. On-line HPLC–CF-LSI-MS analysis of disulfiram photolysis products. Ion current; (A) m/z 212; (B) m/z 281; (C) m/z 297 and (D) m/z 313. (E) HPLC chromatogram of the mixture of photolysis products (UV detection at 225 nm).

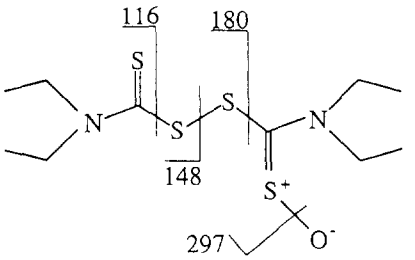

Fig. 4. Proposed structure of the sulfine photolysis product at a HPLC retention time of 9.5 min with assignment of product ions produced after collision induced dissociation of the precursor ion $MH^+ = 313$.

ion at m/z 313 which constituted $<5\%$ of unphotolyzed DSF (as determined by UV) yet completely inhibited ALDH. The molecular ion value indicated addition of a single oxygen to the parent compound DSF without concomitant loss of a hydrogen atom. The product ion spectrum revealed ions at m/z 297 (10%), 180 (55%), 148 (15%) and 116 (10%) (values in parenthesis represent product ion abundance as a percentage of the MH^+ ion). The product ion spectrum strongly indicated the formation of a sulfine derivative of DSF had been formed, as shown in Fig. 4, and further studies are underway to confirm this structure. It is noteworthy that a structurally similar sulfine, which is also an inhibitor of ALDH, has been identified recently as a novel metabolite of DSF [5].

4. Conclusions

HPLC–LSI-MS–MS is a profitable technique for performing on-line structure–activity analysis. While HPLC–LSI-MS is typically considered to be limited by the splitting of sample and limited sample flow, we have found this feature to be useful for probing structure–activity relationships using an on-line analytical technique. By recovering the "waste" eluent from the HPLC–CF-LSI-MS interface, it is possible to closely couple the mass spectral identification of potentially labile photolysis products of DSF with their inhibitory activity against ALDH in vitro.

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