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SHORT COMMUNICATIONS

Bis-(diethyldithiocarbamato) copper complex: a new metabolite of disulfiram?

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The *in vivo* metabolism of disulfiram (DSF) in humans has not yet been completely elucidated [1]. Earlier studies [2, 3] suggest that the rapid reduction of DSF to diethyldithiocarbamate (DDC) is mediated by the glutathione reductase system of the erythrocytes under formation of mixed disulfides with protein sulfhydryl groups. The DDC is further metabolized in part to carbon disulfide and diethylamine and some is excreted after conjugation with glucuronic acid [4]. The DDC is also methylated by microsomal *S*-methyltransferase [5] to form a methyl ester of diethyldithiocarbamate (Me-DDC), a main metabolite found in human plasma.

The aim of the present study was to find metabolic reactions explaining not only the fact that unchanged disulfiram could not be detected in human plasma [11], despite being so reported by some authors [9], but also that only low levels of the main metabolite DDC, are found in plasma from alcoholics after oral administration of 400 mg DSF (Antabus®) [11].

In vitro studies demonstrated that DSF was completely reduced to DDC almost immediately when the compound was added to fresh blood, serum or plasma. The thiol group reacted with cupric ions bound to plasma proteins to form a bis-(diethyldithiocarbamato) metal complex of copper ($Cu(DDC)_2$). The identity of the latter was verified by comparing its retention volume with that of a synthetic product in a high performance liquid chromatography (HPLC) assay and by mass spectrometry (MS).

Materials and methods

Disulfiram (Antabus®) and diethyldithiocarbamate were kindly supplied by Dumex Ltd. (Copenhagen, Denmark). Bis-(diethyldithiocarbamato) copper complex was synthesized according to the method described by Tompsett [6]. Methyl glyoxal, glutathione and glyoxalase I (2000 units/ml) were obtained from Sigma Chemical Company (St. Louis, MO) and 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) from BDH Chemicals Ltd. (Poole, U.K.). All other chemicals were of analytical grade and obtained from E. Merck (Darmstadt, F.R.G.).

A JEOL DMS 300 mass spectrometer equipped with a direct sample inlet probe and interfaced to an Antec Gas Chromatograph was used to confirm the structure and purity of synthetic $Cu(DDC)_2$ and to confirm the identity of the metal complex as an *in vitro* metabolite in human serum and plasma.

MS conditions were: filament 4.8 A, electron voltage 70 eV, ion source temperature 230° and ion multiplier 1.5 kV. The direct inlet probe was programmed up to a final temperature of 400° and with a heating rate of 100°/min.

Plasma copper concentrations were determined in duplicate specimens by atomic absorption spectrophotometry [14] with a Varian AA 775 instrument. To determine the partition coefficient of $Cu(DDC)_2$ between 1-octanol and water the method of Hansch [15] was used. In the HPLC assay disulfiram and its metabolites were separated and quantified on a reversed phase column after on-line precolumn enrichment from directly injected heparin plasma. Plasma DDC was quantitatively analyzed as its ethyl ester derivative by adding 1 μl of ethyliodide, 1 μl of 2-mercaptoethanol and 10 μl of 0.01 moles/l phosphate buffer, pH 7.5, containing 0.2 moles/l EDTA to 1 ml plasma [11]. DSF was added to fresh human heparin blood, serum, heparin-, EDTA, and citrate plasma to a final concentration of 5 nmoles/ml and maintained at 24°. The reduction of DSF to DDC and the formation of $Cu(DDC)_2$ from cupric ions and DDC were continuously followed by analyzing the compounds with the HPLC assay. Spiked samples were all chromatographed before and 1, 2, 4 and 20 hr after preparation.

In order to verify the formation of $Cu(DDC)_2$ as a function of DSF concentration DSF was added to fresh heparin plasma drawn from both alcoholics and a non-alcoholic reference group at concentrations ranging from 100 to 800 nmoles/l. HPLC analysis was performed after a reaction time of 1 hr at ambient temperature.

Prior to MS, aliquots of the eluent containing $Cu(DDC)_2$ were collected from the analytical HPLC column. The eluent was transferred to a glass vial and evaporated to dryness. The residue was dissolved in 25 μl of chloroform, and 5 μl of the solvent was injected into the open-end capillary tube of the direct sample inlet probe. Full mass spectra were obtained by scanning the magnet from a mass of 10 to 1000. Heparin plasma from four alcoholics who had received a single 400 mg dose of DSF (Antabus®) was tested by the HPLC assay for the presence of $Cu(DDC)_2$. Samples were drawn before and 1, 2, 4, 8, 16 and 24 hr after medication.

In order to investigate the effects of DSF administration on the level of reduced erythrocyte glutathione and on the sulfhydryl content in blood and plasma, the glyoxalase I method of Akerboom [7] and the DTNB test for sulfhydryl groups of Ellman [8], were used on the above mentioned samples.

Results and discussion

The addition of DSF to both blood serum and plasma resulted in an immediate and complete reduction of DSF to form an apparent concentration of DDC, probably bound to a copper-containing plasma protein. The time between preparation of the sample and analysis was less than 5 min, yet the content of DSF was below the detection limit of the HPLC assay. Initially, all samples contained 5 nmoles/ml of DSF which was completely reduced to DDC under simultaneous formation of a stoichiometrical molar concentration of $Cu(DDC)_2$. The formation of $Cu(DDC)_2$ from protein-bound cupric ions and DDC is time dependent (Fig. 1) and maximum concentrations were reached almost immediately in blood, 1 hr after preparation in heparin- and citrate-plasma and after 2 hr in serum. The rapid decline in blood may be the result of a distribution of $Cu(DDC)_2$ from plasma into the blood cells. In EDTA plasma, EDTA competes with DDC for cupric ions resulting in a rapid decline of the concentration of $Cu(DDC)_2$.

When comparing the formation of free DDC (assessed as an ethyl ester derivative) with the stability of the plasma concentrations of $Cu(DDC)_2$ with and without addition of the alkylating reagents to the same plasma sample (Fig. 2), it is obvious that the metal complex decomposes faster than the free DDC. The thiol formed is quantitatively converted to the ethyl ester analog of DDC.

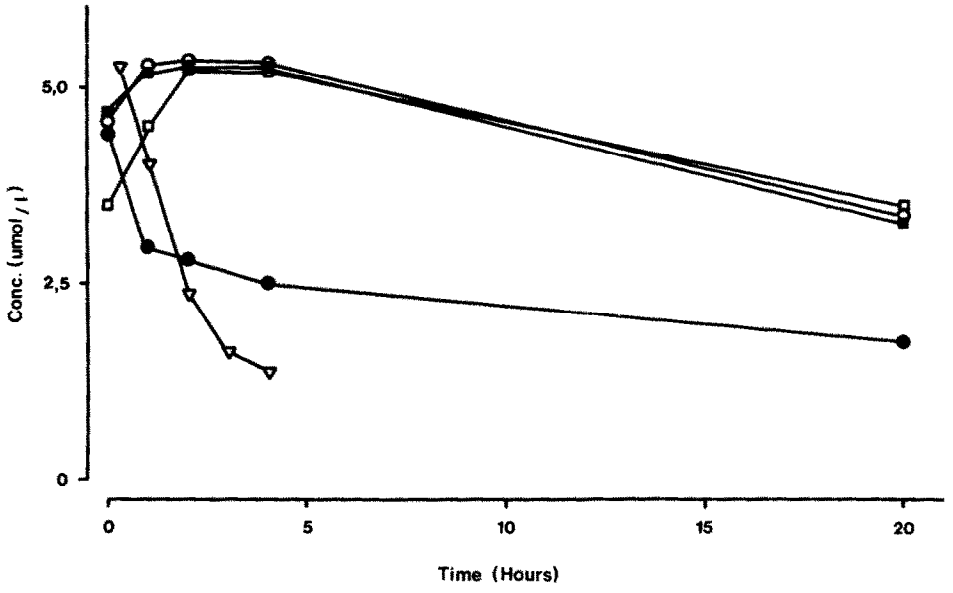

Fig. 1. Formation of $Cu(DDC)_2$ in fresh heparin blood (▽), serum (□), heparin-, (■), citrate- (○) and EDTA plasma (●) at 25° to which DSF (5 μmoles/l) has been added.

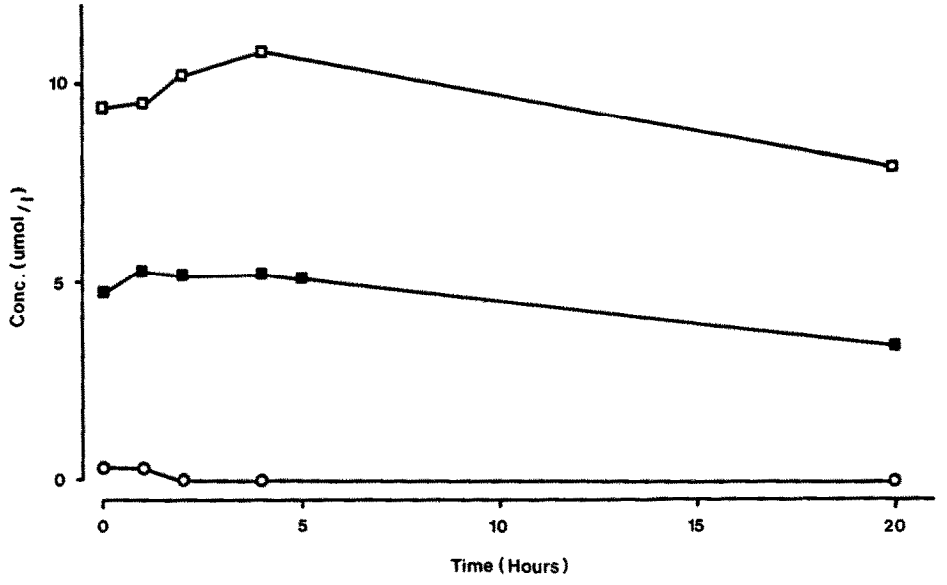

Fig. 2. Formation of $Cu(DDC)_2$ in fresh heparin plasma according to figure 1 (■). Decomposition of $Cu(DDC)_2$ in fresh heparin plasma (○) and formation of ethyl-DDC (□) to which alkylating reagents have been added.

From Fig. 3 an apparent interpatient variability in both groups can be seen for the formation of $Cu(DDC)_2$ after addition of increasing amounts of DSF to plasma. Comparison of alcoholics to non-alcoholics shows a complete overlap (and the difference between them is not significant). Plasma copper concentrations within the groups (mean ± S.D.) were 24.5 ± 6.9 μmoles/l (N = 10) for alcoholics and 26.5 ± 7.4 μmoles/l (N = 4) for non-alcoholics. The individual differences in plasma copper-containing protein capacity to bind DDC does not obviously depend on individual differences in the total plasma copper concentrations [13]. These results suggest that besides an extremely rapid and quantitative reduction of DSF to form DDC the latter also combines quantitatively with cupric ions bound to plasma proteins to form $Cu(DDC)_2$. Cuprous ions are probably oxidized to cupric ions by DSF. It is also suggested that the reaction is concentration dependent, i.e. at a low plasma concentration of DSF there exists an intermediary plasma DDC–Cu–protein complex. When the DSF concentration increases an individual capacity to form $Cu(DDC)_2$ appears.

Mass spectroscopic studies of the HPLC eluent fractions showed fragmentation patterns which involved loss of a DDC ligand radical [10] with a base peak at mass 116. The parent molecular ions with masses of 359 and 361, respectively, assigned to a percentile natural isotope abundance of Cu^{63} and Cu^{65} in the ratio of approx. 7:3. The identity of the spectra obtained from plasma spiked with DSF was confirmed by analyzing a pure synthetic product of $Cu(DDC)_2$.

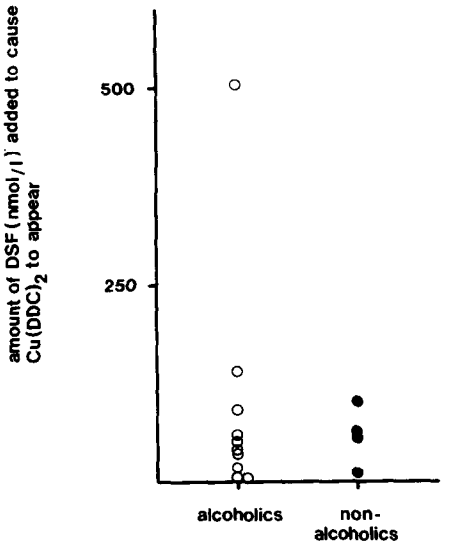

Fig. 3. The appearance of $Cu(DDC)_2$ in fresh heparin plasma drawn from alcoholics ($n = 11$) and a non-alcoholic reference group ($N = 4$) after addition of DSF at concentrations ranging from 100 to 800 nmoles/l.

The plasma concentrations of $Cu(DDC)_2$ in two samples from the *in vivo* studies were found to be just above the detection limit of the HPLC assay. However, the concentration of $Cu(DDC)_2$ found in the eluent fractions collected for measurements by MS was too low to give a definite identification of the compound. The very low plasma concentrations obtained from patients treated with a single dose of DSF could possibly be due to the individual capacity to form $Cu(DDC)_2$ from copper protein bound DDC [11] or to the extremely lipophilic character of the metal complex resulting in its distribution through cell membranes or its deposition into fat tissue. This is in agreement with the recently reported redistribution of endogenous copper into brain tissue when rats and mice were treated with tetramethylthiuram disulfide and DDC, respectively [12, 13]. The $Cu(DDC)_2$ partition between 1-octanol and water was found to be infinite into the organic phase. This finding supports our observation that the compound is highly hydrophobic. Moreover, preliminary results from five patients treated with repeated doses of DSF (400 mg) for 2 weeks show detectable plasma levels of $Cu(DDC)_2$ in all five samples. In the present study no evidence was found that administration of DSF could cause a decrease in the glutathione content of the erythrocytes, nor in the total sulfhydryl activity in blood or plasma. If erythrocyte glutathione was involved in the reduction of DSF, this would probably bring about a decline of the glutathione level in erythrocytes *in vivo* [2].

In summary, the *in vitro* and *in vivo* formation of a bis-(diethyldithiocarbamato) copper complex in humans has been observed. The *in vitro* formation has been clearly verified by MS. However, the plasma levels found *in vivo* are too low to be definitely identified by any available technique and this is probably due to the lipophilic character of the compound.

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Maintenance of cytochrome P-450 in cultured adult human hepatocytes*

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The liver is the primary organ involved in metabolism of xenobiotics. One major metabolic pathway is represented by cytochrome-P-450-dependent mono-oxygenases. Because *in vivo* liver functions are influenced by various endogenous and exogenous factors, a number of investigators have turned to simpler experimental models. Hepatocyte cultures theoretically represent the only *in vitro* model to perform long-term studies in a well-defined environment. However, cultivation of pure populations of adult rodent hepatocytes under various conditions is associated with a selective loss of biotransformation capacity and consequently, to limitation of potential use of this system for pharmaco-toxicological studies [1–3]. Moreover extrapolation of the results obtained with rodent hepatocyte cultures to the human situation has evident shortcomings since qualitative and quantitative interspecies differences commonly exist in biotransformation of xenobiotics by the cytochrome P-450 system [4]. In this study we have measured the cytochrome P-450 content and two

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