Determination of Disulfiram and its Metabolites in Human Blood

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Summary: This work was initiated by the lack of a sensitive method for the determination of disulfiram and its metabolites in blood of patients treated with this drug. A method is described which allows the separate determination of carbon disulfide, free diethyldithiocarbamate and disulfides derived from disulfiram with adequate precision in 10 ml patient blood. It is based on a spectrophotometric determination of a yellow compound formed by trapping carbon disulfide produced from diethyldithiocarbamate and disulfiram in an ethanolic solution of diethylamine and copper(II)-acetate. Good quantitation of disulfiram and diethyldithiocarbamate in blood was achieved by trapping carbon disulfide produced when formic acid and cysteine were added to the samples. During daily administration of 200 mg disulfiram to humans, concentrations of zero to 0.6 µg carbon disulfide and 0.2 to 1.0 µg diethyldithiocarbamate per ml blood were found using this method.

1. Introduction

Disulfiram (tetraethylthiuram disulfide, TETD, Antabus®*) has become a popular drug for treatment of alcoholism since its discovery in 1948 [1]. It is usually administered p.o. in daily doses of 100—400 mg or implanted as tablets containing 1000—1600 mg. The absorption from the gastrointestinal tract seems to be incomplete, 5—20% appearing in the faeces [1—4]; nothing is known on the rate of absorption of implanted disulfiram. Scheme 1 represents the known metabolic pathways of disulfiram or its reduced form diethyldithiocarbamate (DDC). Using 35S-labelled TETD Strömme demonstrated the in vitro and in vivo formation of mixed disulfides with serum or liver proteins [5—9]. No free, unmetabolised TETD could be detected by various authors in serum, liver or urine of experimental animals and man [1, 3, 7, 8]. After i.p. application to rats free DDC could be detected in serum only for a short time and mixed disulfides were formed with protein, indicating rapid interconversions between DDC, TETD and mixed disulfides in vivo. TETD and DDC are further metabolized to CS2 [10, 11], sulfate [7, 10] and DDC-S-glucuronide [7, 12]. A significant part of administered TETD is eliminated in expired air as CS2 [10, 13].

In view of a wide-spread therapeutic use of disulfiram in general and an increased application of the implantation technique it was of interest to determine TETD and its metabolites in patients. Several methods for the determination of disulfiram have been proposed [3, 14—16]. These methods rely on the formation of a yellow copper — TETD — compound which is subsequently extracted with an organic solvent and determined photometrically. When these methods were applied to samples of human blood in our laboratory they proved to lack the necessary sensitivity. High blank values restrict their application to concentrations of TETD or DDC far above those expected in patients. Furthermore they do not account for the formation of mixed disulfides and CS2. Similar problems were encountered when we utilized the recently proposed colour-reaction of TETD with ethanol and cyanide [17]. Hence a method was developed which allows to determine CS2, free DDC and DDC liberated from disulfides (mixed disulfides and/or TETD).

Scheme 1: Main pathways of disulfiram metabolism.

2. Materials and methods

All reagents were of the highest commercial grade from Fluka, Buchs, Switzerland, or Merck, Darmstadt, Germany, and used without further purification. The Viles-reagent was modified as follows: Cu(II)-acetate 0.1 g, diethylamine 35 g, triethanolamine 10 g, demineralised water 75 ml, ethanol abs. ad 1000 ml. Excess solvent was removed by the use of a rotary evaporator Rota-vapor Büchi, Flawil, Switzerland. Solutions were filtered with a syringe-filter apparatus from Sartorius (Göttingen, Germany) with solvent resistant and inert cellulose membrane filters SM 11 604. The spectra were recorded on a Zeiss PMQ II and absorbances determined on a Unicam Sp 1800.

3. Results and discussion

3.1. Evaluation of published methods

Previous methods [3, 14—16] to determine TETD and DDC were based on the fact that these compounds form yellow complexes with cupric ions. Possible structures of these have been reported by Geldmacher et al. [18]. Although the precise nature of these copper-addition compounds is not yet known, they will be referred to as copper-complexes. From their study these authors concluded that the complexes formed from DDC and TETD cannot be distinguished on the bases of several physico-chemical parameters. As shown in Fig. 1, identical spectra were obtained by the addition of copper(II)-acetate to DDC and TETD in two solvent-systems. For the formation of the TETD-complex the samples were heated to 50°C for 15 min to enhance the rate of complex formation. As reported previously [14, 18], two absorption maxima are observed at 266 nm and 400 nm in ethanol with molar extinction coefficients of 25 000 and 7200, respectively. The addition of diethylamine to DDC and TETD decreases with increasing pH. Since this finding could not be reproduced attempts were made to separate the free compounds before formation of the copper complex. As suggested by Domar et al. [3], DDC is ionized at pH 9 and should not be extractable with a lipophilic solvent in contrast to TETD. Subsequent adjustment to an acid pH should allow extraction of DDC. Attempts to separate DDC and TETD in this way failed because DDC was found to contaminate TETD by approximately 30% at pH 9. Furthermore DDC decomposes to CS₂ and diethylamine both in alkaline and acidic solution. Identification of TETD by means of thin-layer chromatography has been proposed by Farago [16]. However, a separation and quantitation of TETD and DDC could not be achieved by this technique. Although the two compounds were separated with CHCl₃, benzene 18, methanol 15, butanol 10, NH₃ 25% 2 (vol. parts) as solvent system, such a procedure could not be used for subsequent quantitation due to losses of DDC by decomposition.

3.2. Determination of CS₂ and DDC

Since CS₂ has been identified as a major metabolite of TETD [10, 11, 13, 19] it seemed appropriate to establish a method for its determination in human blood. DDC decomposes rapidly and quantitatively to CS₂ and diethylamine in acidic solution and hence can be determined by measuring the CS₂ evolved [20—22]. A special apparatus was employed (Fig. 2) for this purpose.

![Fig. 1: Spectra of disulfiram-, DDC- and CS₂-copper complexes](image1)

![Fig. 2: Apparatus employed for the determination of CS₂, DDC and disulfiram.](image2)
this procedure and for at least 24 h at room temperature in solution. Absorbance was determined with 4 cm path-length at 426 nm; measurements at 270 nm were less reliable because of high reagent blanks. A molar extinction coefficient of 13,120 was obtained by adding CS$_2$ directly to the reagent in the trap and using the procedure described (see below). Hence, a concentration of 1 μg CS$_2$/ml sample should yield an extinction corrected for reaction blank of 0.862.

3.3. The calibration curve for CS$_2$

A calibration curve for CS$_2$ was obtained by adding various amounts of CS$_2$ to 10 ml samples of urine, serum and blood (Fig. 3). The absorbance of 0.866 for a sample concentration of 1 μg CS$_2$/ml calculated from the slope of the calibration curve is in good agreement with the value obtained from the molar extinction coefficient; these results indicate a full recovery of CS$_2$ added to blood, serum and urine in a range of 0.05 to 1.5 μg/ml.

![Fig. 3: Calibration curve for CS$_2$. Various amounts of CS$_2$ added to 10 ml blood (●), serum (▲) or urine (●) were analysed as mentioned in 3.6.1. The absorbance is corrected for the reagent blank. y = 0.866x + 0.014; R = 0.998.](image)

3.4. The calibration curve for DDC (Fig. 4)

Various amounts of DDC were added to blood, serum and urine and the CS$_2$ evolved from the addition of formic acid was determined as described. One mole of CS$_2$ could be recovered from one mole of DDC added to urine. However, recoveries varying between 30 to 80% were found with serum and virtually no CS$_2$ was liberated from DDC added to blood under these conditions. Since free DDC cannot form mixed disulfides it seems that it is oxidized to TETD. By addition of cystein to reduce disulfides full recovery was obtained. 1 μg CS$_2$/ml is equivalent to 1.96 μg DDC/ml. For this DDC concentration of 1.96 μg/ml an extinction of 0.875 could be calculated from the slope of the calibration curve. This is in good agreement with the value obtained for CS$_2$.

![Fig. 4: Calibration curve for DDC. Various amounts of DDC-Na added to 10 ml blood (●), serum (▲) or urine (●) were analysed as mentioned in 3.6.1. The absorbance is corrected for the reagent blank. y = 0.447x — 0.006; R = 0.997.](image)

3.5. The calibration curve for TETD (Fig. 5)

Cystein and formic acid were added to the samples of blood, serum and urine containing TETD (Fig. 5). No CS$_2$ formation was detectable without cystein, confirming that no reduction of TETD took place. This method does not discriminate between TETD and possibly formed mixed disulfides. Again good agreement was found between the slopes of the calibration curve for TETD with that for CS$_2$. Since it has to be considered that 2 moles CS$_2$ are formed from one mole of TETD, 1 μg CS$_2$/ml corresponds to 1.95 μg TETD/ml and yields an extinction of 0.850.

![Fig. 5: Calibration curve for TETD. Various amounts of TETD added to 10 ml blood (●), serum (▲) or urine (●) were analysed as mentioned in 3.6.1. The absorbance is corrected for the reagent blank. y = 0.436x — 0.002; R = 0.997.](image)

3.6. Determination of CS$_2$, DDC and TETD in the same sample

Various amounts of all three compounds were added to water to yield mixtures within the range of concentrations used in the calibration curves. Free CS$_2$ was flushed and trapped in a first reagent solution. CS$_2$ subsequently liberated from DDC by addition of formic acid was captured in a new trap. Finally the remaining TETD was reduced with cystein and determined like DDC. All three compounds could be recovered separately within the accuracy obtained in the calibration curve.

3.6.1. Procedure

Mix 10 ml blood, serum or urine with 20 ml water and 0.1 ml n-octanol (antifoaming agent). Flush free CS$_2$ with N$_2$ at room temperature for 30 min with 100 ml N$_2$ per min. Trap the CS$_2$ in 6 ml of the modified Viles reagent (see 2.). Transfer the yellowish reagent solution into a flask and rinse the trap successively with 3 ml ethanol and 5 ml benzene. Evaporate the combined solution at 50°C and 15 Torr. Dissolve the residue in 2 ml of a mixture of methylacetate-acetone (7:3 vol.), filter and read the absorbance at 426 nm in 4 cm cuvettes using the solvent mixture as the reference.

To determine free DDC and disulfides use a new trap with fresh reagent and add 5 ml formic acid and 0.1 g cystein-HCl. Heat
Table 1 shows the results of within-day estimates of precipitation and recovery for 3 different concentrations of DDC-Na added to blood samples and for reagent blanks obtained from different blood samples or water. The same values for the reagent blank were obtained when various blood samples were used instead of water. The reagent blanks showed a relatively large variation but remained constant for one batch of reagents and for solutions stored for 2 weeks. A CV of 21 was found for a DDC-Na concentration of 0.1 µg/ml blood. At these low blood levels the method is limited by the large contribution of the blank to the absorbance values of tests. Table 2 shows the good recovery of CS₂ and DDC-Na added simultaneously to various blood samples.

3.7. Within-day precision and recovery

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3.8. Analyses of patient blood

In order to test the practicability of the method within the range of concentration of CS₂ and total DDC in the blood of patients, daily doses of 200 mg TETD were administered between 8 and 10 p.m. to three individuals and blood was taken at 8 a.m. for analysis. The results are shown in Fig. 6. CS₂-concentrations ranged from zero to 0.6 µg/ml and DDC-concentrations from 0.2 to 1.0 µg/ml. It is of interest to note that the CS₂-concentrations varied over a broad range simultaneously in all these subjects. Although the reason for this phenomenon is not known, it seems to be of physiological origin, since experimental errors were excluded by analyses of standard solutions.

4. References


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