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Determination of sodium diethyldithiocarbamate (Imuthiol) and its S-methyl metabolite by gas chromatography–mass spectrometry

Use of deuteromethyl iodide derivatization

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ABSTRACT

A gas-chromatographic–mass spectrometric method is described to measure the plasma concentration of sodium diethyldithiocarbamate (ditiocarb sodium, DEDTC-Na), the active ingredient of Imuthiol®, a drug found to be active in the opportunistic infections occurring in AIDS, and its S-methyl metabolite. Plasma samples are treated with deuteromethyl iodide and DEDTC-Na is transformed into its deuteromethyl ester, which is then co-extracted with the S-methyl metabolite. Gas chromatography–mass spectrometry and selected-ion monitoring allow the specific determination of both compounds. Linear calibration curves were obtained up to 4000 ng/ml This method has been succesfully applied for pharmacokinetic studies after Imuthiol and disulfiram, the dimer of DEDTC, were administered to humans

INTRODUCTION

Imuthiol® (sodium diethyldithiocarbamate, ditiocarb sodium, DEDTC-Na) has been developed as a drug and found to be active in the opportunistic infections occurring in AIDS patients. It is used once a week either orally (enteric-coated capsules) at a dose of 10 mg/kg or intravenously (5 mg/kg).

DEDTC-Na is a very labile molecule in acidic media, in which it decomposes rapidly into diethylamine and carbon disulphide. After absorption, besides this spontaneous decomposition, DEDTC-Na undergoes conjugation with glucuronic acid and is excreted in urine as DEDTC-glucuronides [1]. Methylation of DEDTC occurs in the liver and the kidneys, resulting in DEDTC methyl ester (DEDTC-CH_3) [2], transformed into methylmercaptan and then formaldehyde and inorganic sulphates. It has been demonstrated that a small proportion could be oxidized to disulfiram, of which DEDTC is the first metabolite.

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Since the observation by Hald *et al.* [3] of the ethanol-sensitizing effect of disulfiram (tetraethylthiuram disulphide), this drug has found considerable acceptance in formating part of an aversion therapy for treating alcoholics. Disulfiram can be considered to be a dimer of diethyldithiocarbamic acid. After absorption in animals and humans, it is transformed by reduction into diethyldithiocarbamic acid [4]. The metabolic pathway is then the same as that of sodium diethyldithiocarbamate.

Analytical methods used for disulfiram assay, other then the radioactive one using ^{35}S, are based on the production of a coloured cupric complex [5,6] with the reduction product of disulfiram or diethyldithiocarbamic acid. These colorimetric procedures lack the specificity needed to discriminate between disulfiram and its other metabolites. A gas chromatographic (GC) procedure has been developed by Cobby *et al.* [7] in which DEDTC is transformed into its methyl ester by methyl iodide before GC analysis. A determination of disulfiram and its metabolites from biological fluids by high-performance liquid chromatography has been developed, based on the quantitative stepwise extraction of disulfiram and back-conversion of the various metabolites into DEDTC-CH_3 [8].

In order to compare the pharmacokinetic and metabolic behaviour of Imuthiol and disulfiram, it was essential to measure the plasma levels of both DEDTC and its main metabolite, DEDTC-CH_3. For this purpose we developed a gas chromatographic–mass spectrometric (GC–MS) method that was able to determine simultaneously from the same sample the two compounds after the derivatization of DEDTC into DEDTC-C^2H_3 with IC^2H_3. Fig 1 shows the principle of this method. Owing to its poor lipophilicity, DEDTC cannot be extracted from plasma by the usual organic solvents whereas DEDTC-CH_3 is quite easily extracted. When a plasma sample containing DEDTC and DEDTC-CH_3 is treated

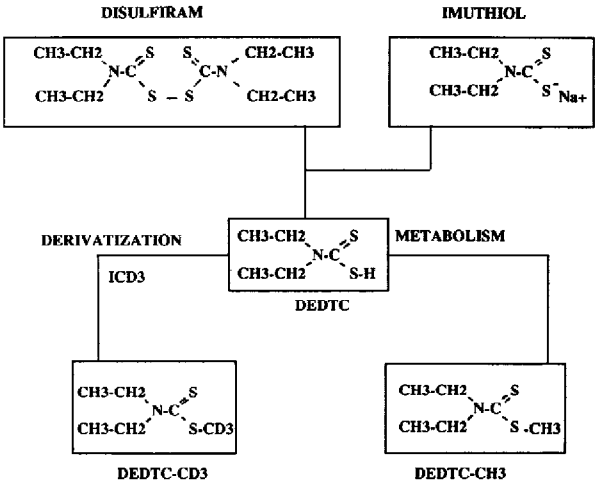

Fig 1 Principle of the method derivatization and metabolism

with IC^2H_3, DEDTC is transformed into DEDTC-C^2H_3 (the deuteromethyl ester), which can be extracted with its methylated counterpart in the same operation with the same efficiency. It is then possible to specifically measure both compounds using GC–MS and selected-ion monitoring (SIM).

EXPERIMENTAL

Reagents and chemicals

Ethanol (RPE-ACS grade, Carlo Erba, Milan, Italy) was used to prepare DEDTC-Na, DEDTC-CH_3 and diethyldithiocarbamate ethyl ester (DEDTC-C_2H_5) stock and working solutions. Hexane (RPE grade, Carlo Erba) was used as extraction solvent.

Disodium ethylenediaminetetreaacetate trihydrate (analytical grade, Riedel de Haen, Hannover, F.R.G.) was used to prepare the EDTA–NaCl solution. Sodium diethyldithiocarbamate trihydrate, the active ingredient of Imuthiol, was obtained from Merck (Darmstadt, F.R.G.). The deuteromethyl iodide used for DEDTC derivatization was purchased from Cambridge Isotope (Innerberg, Switzerland).

The internal standards, diethyldithiocarbamate ethyl ester and diethyldithiocarbamate methyl ester, were synthesized in our laboratory.

The synthesis of these two esters was performed according to the method described by Cobby *et al.* [7]. DEDTC-C_2H_5 was prepared by adding ethyl iodide (20 ml) to 40 g of DEDTC-Na in 100 ml of absolute ethanol The mixture was cooled during the slow addition of ethyl iodide The solution was then kept under magnetic stirring at ambient temperature and protected from light for 60 min. The lower oily yellow layer was withdrawn and dried over anhydrous calcium chloride for 24 h. After filtration the light yellow liquid was fractionally distilled under reduced pressure (5 mmHg) and the fraction boiling at 118–119°C was collected and stored at 4°C over calcium chloride.

Diethyldithiocarbamate methyl ester was prepared according to the same procedure by slow addition of methyl iodide (16 ml) to an ethanolic solution of DEDTC-Na (40 g per 100 ml). The fraction boiling at 117–118°C under a reduced pressure of 8 mmHg was collected. The purity of the products was assessed by GC–MS analysis and NMR spectroscopy

Stock solutions

DEDTC-Na, DEDTC-CH_3 and DEDTC-C_2H_5 stock solutions were prepared by dissolving 100 mg of each compound into 100 ml of ethanol. They were stored at 4°C and were stable for one month. They were used to prepare fresh working solutions just before use.

EDTA–NaCl solution

This solution was prepared by dissolving 7.44 g of sodium ethylenediamine-

tetraacetate trihydrate in 100 ml of distilled water. Then 100 g of NaCl were added and the pH of the resulting solution was adjusted to 8.5 with NaOH. The volume was then made up to 1000 ml with distilled water.

Derivatization and extraction procedures

A 1-ml volume of the EDTA–NaCl solution was added to 1 ml of plasma and 100 μl of IC^2H_3 were added. After 5 min shaking, the mixture was left at ambient temperature and protected from light for 15 min. The internal standard (DEDTC-C_2H_5) was added at a concentration of 1000 ng/ml and mixed on a vortex-mixer for 10 s. After addition of 7 ml of hexane, the samples were extracted using an automatic shaker for 10 min. After centrifugation, the organic phase was recovered and evaporated under a stream of nitrogen in an ice-bath. The evaporation was stopped when the volume was reduced to *ca.* 50 μl. The residues were kept at -20°C until GC–MS analysis.

Chromatographic conditions

The gas chromatograph was a Hewlett Packard Model 5790, equipped with an OV 1701 fused-silica capillary column (15 m × 0.25 mm I.D.) (Hewlett Packard, Les Ullis, France). Samples (1–2 μl) were injected in the splitless mode (valve time, 0.75 min). The injector port was set at 140°C and the oven temperature was raised from 90°C (0.8 min) to 210°C (0.9 min) at 20°C/min. The transfer line between the gas chromatograph and the mass-selective detector was set at 260°C. Helium was used as carrier gas with a head pressure of 1 bar.

Detection and measurements

A Hewlett Packard mass-selective detector (HP 5970A) operating between 3 and 4.2 min after the start of the analysis was used for detection. The monitored ions were m/z 163 for DEDTC-CH_3, m/z 166 for DEDTC-C^2H_3, and m/z 177 for DEDTC-C_2H_5.

Mass spectrometer operations and data treatment were controlled by an HP 59970 MS Chemstation.

RESULTS

Chromatographic separation and mass spectra

Fig. 2 shows the fragmentogram corresponding to the monitored ions at m/z 163, 166 and 177. These three chromatographic peaks with respective absolute retention times of 3.41, 3.43 and 3.83 min, correspond to DEDTC-C^2H_3, DEDTC-CH_3 and DEDTC-C_2H_5 (internal standard). A slight isotope effect on the retention times can be observed between DEDTC-CH_3 and DEDTC-C^2H_3, due to the deuterium substitution of the methyl group.

Fig. 3 shows the mass spectra of (a) DEDTC-CH_3, (b) DEDTC-C^2H_3 and (c) DEDTC-C_2H_5. The m/z values for the molecular ions, which correspond to the

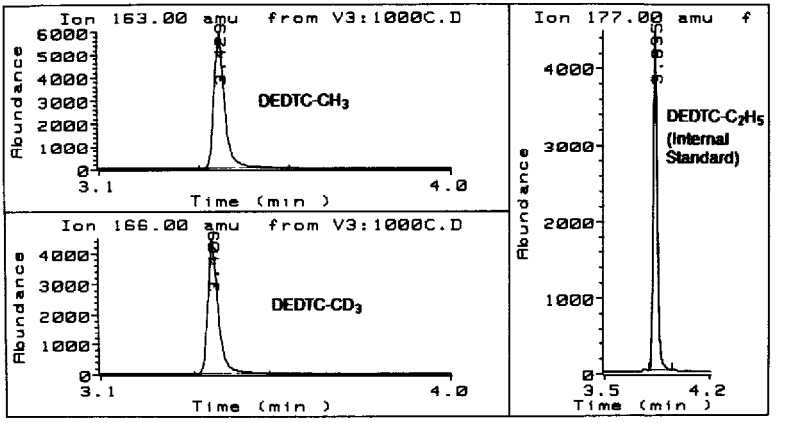

Fig 2 Fragmentogram of the monitored ions DEDTC-CH_3, m/z = 163; DEDTC-CD_3, m/z = 166, DEDTC-C_2H_5, m/z = 177 (I.S.)

monitored ions, are at 163, 166 and 177, respectively. Fig 4 shows the mass spectrum recorded on the DEDTC chromatographic peak from the plasma of a dog, taken 3 min after Imuthiol intravenous administration. Ions at m/z 163 and 166 can be observed; they correspond to the S-methylated metabolite of DEDTC and to DEDTC itself, measured as its deuteromethyl ester derivative.

Linearity

Various plasma samples spiked with DEDTC-Na and DEDTC-CH_3 at concentrations of 50, 100, 500, 1000, 2000, 3000 and 4000 ng/ml were prepared and treated as described above. A linear regression analysis of the values (peak-area

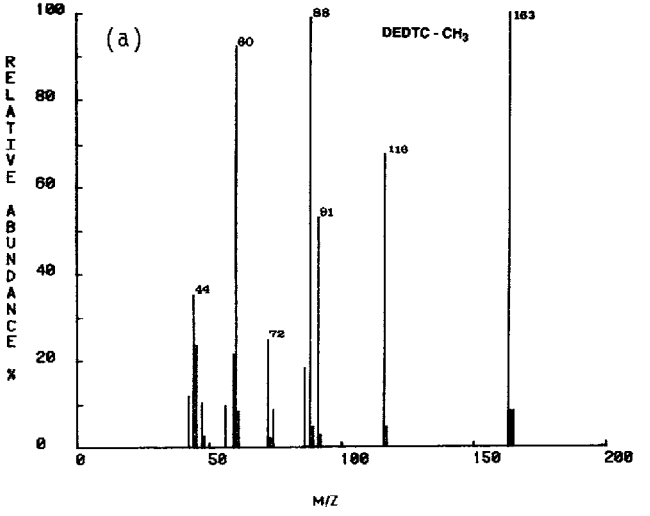

Fig 3.

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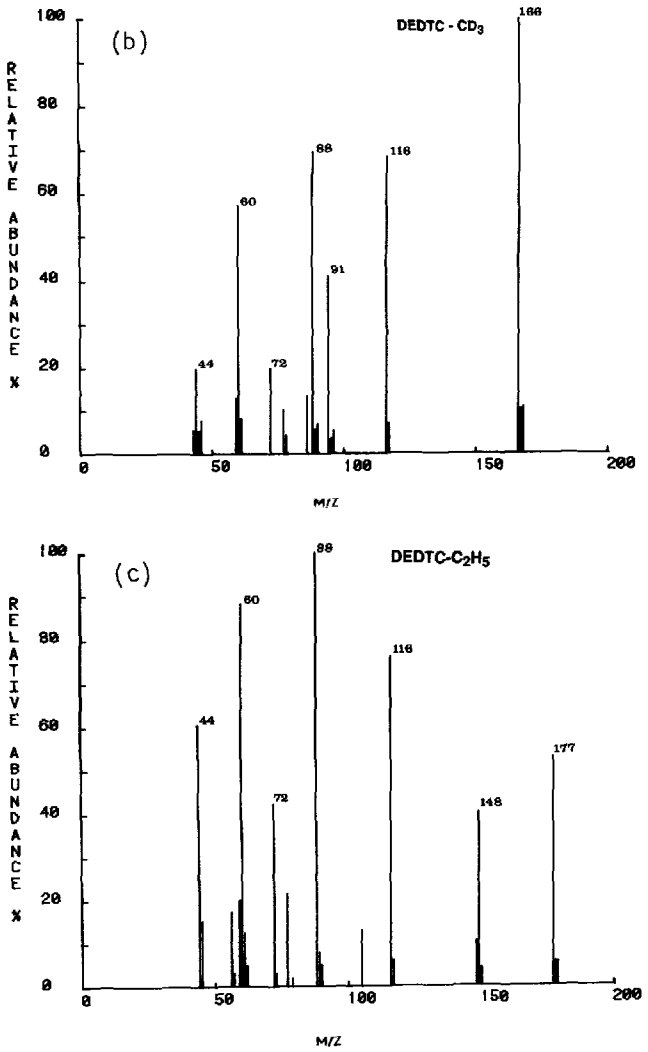

Fig. 3 Mass spectra of (a) DEDTC-CH_3, (b) DEDTC-CD_3 and (c) DEDTC-C_2H_5

ratios *versus* concentration) indicated a linear fit of the data. DEDTC-Na as DEDTC-C^2H_3: slope, 1.15; intercept, 0.076, $r^2 = 0.998$. DEDTC-CH_3: slope, 1.44; intercept, -0.004; $r^2 = 0.999$.

Precision

The precision of the method was estimated by the measurement of both the repeatability and the reproducibility within the therapeutic range. The repeatability was studied by measuring the DEDTC-Na and DEDTC-CH_3 concentrations from five plasma samples spiked with 200 and 2000 ng/ml of each compound,

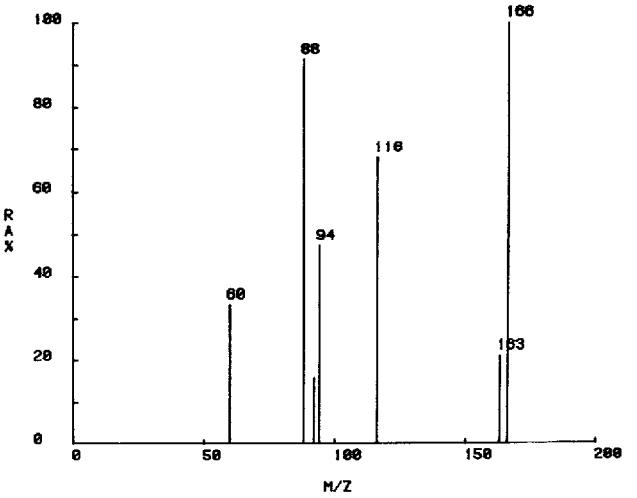

Fig. 4 Mass spectrum from a plasma extract. Molecular ions of DEDTC-CH_3 and DEDTC-C^2H_3 can be observed at *m/z* 163 and 166.

TABLE I

REPEATABILITY OF THE METHOD

Sample No	Area ratio	
	DEDTC-CH_3/DEDTC-C_2H_5	DEDTC-C^2H_3/DEDTC-C_2H_5
Sample concentration 200 ng/ml		
1	0.274	0.195
2	0.275	0 207
3	0.272	0.216
4	0.280	0.181
5	0 274	0.210
Mean	0 275	0.201
S D.	0 003	0.013
C V (%)	1 09	6 89
Sample concentration. 2000 ng/ml		
1	2.914	2 672
2	2 923	2 864
3	2 888	2 423
4	3 009	2.691
5	2 897	2 755
Mean	2 927	2 681
S.D	0.048	0 162
C.V. (%)	1 64	6 06

respectively. These measurements were performed on the same day and under the same experimental conditions. The results are reported in Table I.

The reproducibility was evaluated by the same procedure as the repeatability, but over a five-week period. The range of concentrations was 500–2000 ng/ml. The mean coefficient of variation (C.V.) for DEDTC-CH_3 determination was between 2.8 and 7.9% within the range of concentration. For DEDTC-Na determination (as DEDTC-C^2H_3) the mean C.V. over the whole range was 15%, taking in account the derivatization process.

Accuracy

The comparison of the measured concentration against true (or target) concentrations in the range 500–2000 ng/ml for fifteen different spiked plasma samples gave the following results. DEDTC-Na as DEDTC-CD_3: slope 1.043 ± 0.046 (not significantly different from 1); intercept, not significantly different from zero; $r^2 = 0.978$. DEDTC-CH_3: slope, 1.014 ± 0.023 (not significantly different from 1); intercept, not significantly different from zero; $r^2 = 0.994$.

Limit of detection

The limit of detection was taken as the lowest concentration yielding an integrated area corresponding to ten times the area measured in the same integration window after injection of an extract from a blank plasma. For DEDTC-Na and DEDTC-CH_3 this limit was *ca.* 3 ng/ml

Example of human kinetics

The method was used for the quantitative determination of DEDTC-Na and DEDTC-CH_3 in human plasma after oral administration of either Imuthiol or disulfiram, in order to study and compare the pharmacokinetics of these drugs. For illustration, Fig. 5 shows the DEDTC-Na and DEDTC-CH_3 plasma levels–time curves obtained from a subject after administration of the equivalent of 10 mg/kg DEDTC as Imuthiol enteric-coated capsules (a) and as disulfiram non-enteric-coated capsules (b).

DISCUSSION

The proposed method offer several advantages in term of specificity and the ability to measure DEDTC-Na and DEDTC-CH_3 plasma levels simultaneously. The method is more sensitive and specific than colorimetric methods and simpler than that requiring the conversion of diethyldithiocarbamic acid into CS_2. The use of derivatization with IC^2H_3 and GC–MS with SIM allows the simultaneous extraction and determination of DEDTC-Na as its deuteromethyl derivative and of the main metabolite DEDTC-CH_3, which was not possible with the first GC method described [7], and needed several intermediate operations in the HPLC method [8]. When using IC^2H_3 derivatization and GC–MS measurements this

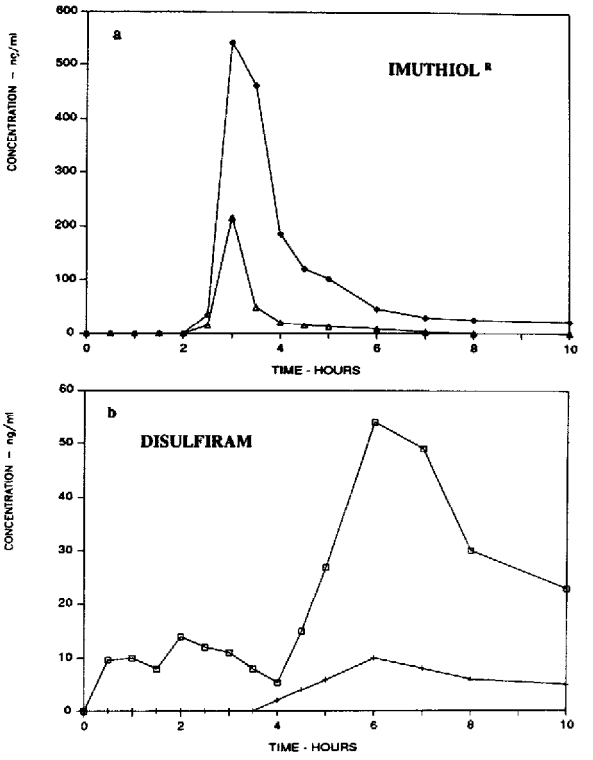

Fig. 5. Plasma concentration–time curves after (a) Imuthiol administration [DEDTC (△), DEDTC-CH_3 (◇)] and (b) disulfiram administration [DEDTC (+); DEDTC-CH_3 (□)]

method exhibits good parameters in terms of analytical quality and practicability.

The extraction method described by Jensen and Faiman [8] using chloroform after addition of an EDTA–1% NaCl solution (pH 8.5) was modified. The NaCl concentration was raised to 10%, and several other solvents were investigated. Chloroform was replaced by hexane in order to obtain cleaner plasma extracts containing fewer interfering impurities, especially cholesterol, which gives an intense chromatographic peak at a rather long retention time and then increases the column pollution and the time of analysis. Heptane also gives clean plasma extracts.

It is important to notice that solvent evaporation is a critical operation and has to be performed in an ice-bath because of the high volatility of DEDTC-CH_3 and DEDTC-C^2H_3, and must not be carried on until dryness.

The proposed method makes it possible to study sodium diethyldithiocarbamate (Imuthiol) pharmacokinetics during pharmacological investigations in AIDS. Because of the short half-life of DEDTC-Na, it is important to determine the plasma levels of the main metabolite, DEDTC-CH_3, whose plasma residence time is longer than that of the parent drug.

The proposed method is able to determine both the drug and the metabolite simultaneously and specifically.

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