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RAPID AND SENSITIVE ON-LINE PRECOLUMN PURIFICATION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR DISULFIRAM AND ITS METABOLITES

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SUMMARY

The disulfiram (Antabus®) metabolites diethyldithiocarbamate, diethyldithiocarbamate methyl ester, carbon disulphide and bis(diethyldithiocarbamato) copper complex were quantitatively analysed from directly injected heparin plasma by reversed-phase high-performance liquid chromatography. The highly volatile metabolite, carbon disulphide, was converted to the methyl ester of dimethyldithiocarbamate before chromatography. The analytical procedure is simple and does not require sample preparation or addition of an internal standard, and the compounds are eluted from the columns in 15 min. After automated on-line precolumn enrichment, the parent compound and biotransformation products could be back-flushed and chromatographed on an ordinary reversed-phase column. The influence of plasma protein binding on the constituents in the precolumn enrichment step was also investigated. Dissociation and partition of constituents from plasma proteins gave a complete retardation on the precolumn. The time courses of diethyldithiocarbamate and its methyl ester were followed in patients receiving therapeutic doses of disulfiram.

INTRODUCTION

Disulfiram (Antabus®; DSF) is a drug used in the treatment of alcoholism in man [1]. Even though the drug has been used for more than 35 years, the degradation mechanisms and the pharmacokinetics of DSF are poorly understood.

So far, only one paper [2] has reported detectable blood concentrations of unchanged drug after DSF administration to alcoholics. The present investigation confirmed the results of other studies [3, 4] that DSF is unstable in human blood and is rapidly and quantitatively reduced to diethyldithio-

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carbamate (DDC). The thiol formed reacted with plasma protein-bound cupric ions in a time- and concentration-dependent reaction. When plasma DDC is in excess, the plasma protein is displaced by the former under simultaneous formation of a bis(diethyldithiocarbamato) copper complex [5].

Previously reported methods for quantitative determination of DSF and its degradation products are based on spectrophotometry [7, 8], polarography [9], radiometric assay [10], gas chromatography [11, 12] and high-performance liquid chromatography (HPLC) [3, 4, 13]. However, these methods are deficient in either speed, sensitivity, reproducibility or simplicity and could not be used when the rapid reduction of DSF to DDC was to be followed in vitro in human plasma. In order to investigate the initial metabolism of DSF in vivo in humans or in vitro in human blood samples, it was found necessary to apply an on-line precolumn purification and enrichment HPLC assay [6].

EXPERIMENTAL

Chemicals

DSF, DDC as its sodium salt and the methyl, ethyl and propyl esters of DDC (Me-DDC, Et-DDC and Prop-DDC) were generous gifts from A/S Dumex (Copenhagen, Denmark).

Bis(diethyldithiocarbamato) copper complex [$Cu(DDC)_2$] synthesized according to Tompsett [8] and dimethyldithiocarbamate (DMDC) was synthesized according to Masso and Kramer [3].

Ethyl iodide and dithiothreitol were obtained from Merck (Darmstadt, F.R.G.) and 2-mercaptocthanol from BDH (Poole, U.K.). All other chemicals were of analytical grade and obtained from Merck.

Blood was collected in Venoject vacuumized sodium heparin collection tubes.

Apparatus

A Model 8500 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with a Model 440 absorbance detector (254 and 436 nm) (Waters Assoc., Milford, MA, U.S.A.), a Model 7125 sample injector (Rheodyne, Cotati, CA, U.S.A.), a Model 7000 switching valve (Rheodyne) connected to a Model 7001 pneumatic actuator (Rheodyne) for automatic operation and a Model Vario Perplex II peristaltic pump (LKB, Bromma, Sweden) for washing the precolumn, were used for HPLC analyses. The purification and enrichment of the sample was performed by a Perisorb RP-18, 40-μm precolumn (5 cm × 3.9 mm I.D.) (Merck) and the isocratic separations by a LiChrosorb RP-18, 7-μm reversed-phase column (25 cm × 3.9 mm I.D.) (Merck), respectively. The mobile phases were mixtures of 0.01 mol/l phosphate buffer (pH 7.5) and acetonitrile in volume ratios of 40:60 for DSF, Et-DDC, Me-DDC, carbon disulphide (Cs_2), DMDC and Prop-DDC and 15:85 for $Cu(DDC)_2$. The precolumn washing solution was a 0.01 mol/l phosphate buffer (pH 7.5) containing 0.005 mol/l EDTA. The flow-rate was 1.0 ml/min for both pumps.

Preparation of standards

Stock solutions of DSF, sodium-DDC, Me-DDC, CS_2, $Cu(DDC)_2$ and the

internal standard (Prop-DDC) in ethanol, phosphate buffer (for sodium-DDC) and chloroform [for $Cu(DDC)_2$] were prepared at concentrations of 10 mmol/l.

Working solutions containing DSF, sodium-DDC, Me-DDC, CS_2 and $Cu(DDC)_2$ were made up in ethanol and/or drug-free, fresh heparin plasma, respectively, at concentrations ranging from 25 to 1500 nmol/l and 25 to 400 μmol/l (for CS_2). The internal standard working solution was prepared at a concentration of 100 μmol/l.

Derivatization procedures

A 10-μl volume of 0.01 mol/l phosphate buffer (pH 7.5) containing 0.2 mol/l EDTA, 1 μl of 2-mercaptoethanol and 1 μl of ethyl iodide was added to a 1-ml plasma sample or working plasma standard solution, placed in 3-ml glass tubes. The tubes were mixed for 30 s and incubated at 40°C for 30 min. A 100-μl plasma volume was directly injected onto the precolumn for on-line enrichment and purification of the solutes before chromatographic separation and detection at 254 nm of DDC as its ethyl ester and of Me-DDC. To determine CS_2, 40 μl of dimethylamine (33% in ethanol), 30 μl of 0.01 mol/l phosphate buffer (pH 7.5) containing 0.2 mol/l of EDTA, 15 mmol/l of dithiothreitol and 5 μl of ethyl iodide were added to a 1-ml plasma sample or working plasma standard solution, and treated as described above, except that the specimens were incubated at room temperature overnight; 20-μl plasma volumes were injected into the chromatograph.

Analysis of unchanged DSF and $Cu(DDC)_2$ was performed in plasma without the addition of alkylating reagents. The plasma injection volumes for detection of DSF and $Cu(DDC)_2$ were 100 and 500 μl, respectively. DSF was monitored at 254 nm, while $Cu(DDC)_2$ was registered at 436 nm.

Chromatography

The precolumn purification HPLC equipment shown in Fig. 1 was used for isocratic separations. The sample compounds of interest were selectively retarded on the precolumn after injection. Water-soluble polar coproducts were simultaneously eliminated and eluted in the waste by solvent from pump I. While washing the precolumn for 4 min, the analytical column was equilibrated in the mobile phase from pump II. To complete the precolumn washing cycle, the switching valve was automatically changed from LOAD to INJECT for another 4 min and was then returned to its original position. Elution of selectively retained parent drug and metabolites was then carried out in the reverse direction to the sample application. The compounds were separated on the analytical column before detection at 254 or 436 nm.

Standard curves

Standard curves were prepared by adding known amounts of DSF, DDC, Me-DDC, $Cu(DDC)_2$ (25—1500 nmol/l) and CS_2 (25—400 μmol/l) to ethanol and/or fresh heparin plasma. DSF, CS_2 and $Cu(DDC)_2$ were determined separately, while DDC and Me-DDC were analysed in the same analysis. The peak areas and, where appropriate, peak-area ratios of standard to internal standard were calculated and plotted against known concentrations. The peak

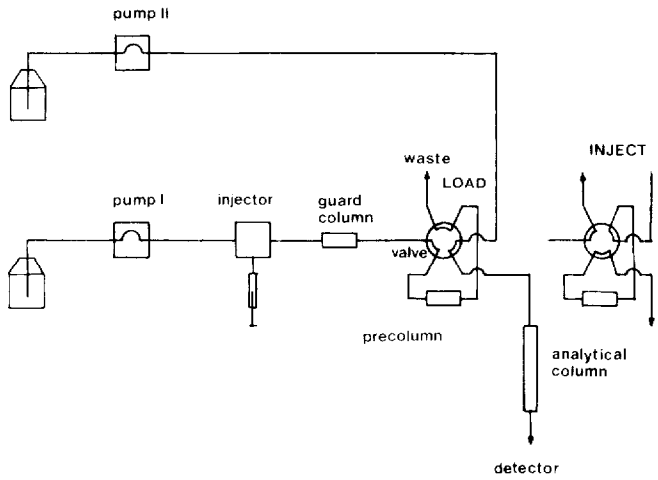


Fig. 1. HPLC system. The precolumn was equilibrated in the washing phosphate buffer from pump I and the analytical column in one of the two mobile phases from pump II, respectively. Aliquots of 20, 100 or 500 μl of plasma standard or sample were directly injected in the loop injector. Solutes were enriched and purified on the precolumn and coproducts were eliminated in the waste in the 4-min precolumn washing cycle. Retarded compounds were eluted, separated and detected in the 4-min elution cycle.

areas of unknown samples were calculated in the same way and compared to the standard curves.

Recovery and precision

The mean recoveries of Me-DDC, DDC and CS_2 were determined at two concentration levels, with and without the addition of internal standard, and after the addition of 100 and 1000 nmol/l (for Me-DDC, DDC) and 50 and 400 μmol/l (for CS_2) to plasma, and compared with the yield found in ethanolic standard solutions.

The precision of the method was tested at the same plasma concentration levels as in the recovery study after addition of Me-DDC, DDC and CS_2 to plasma.

To determine the efficiency of the ethylation and amination reactions, recovery experiments on DDC as its ethyl ester and on CS_2 as the dimethyldithiocarbamate ethyl ester were performed after addition of 1.35 μmol/l sodium-DDC and 0.165 mmol/l CS_2 to heparin plasma and compared with standard solutions made up in ethanol.

Evaluation of internal standards

A 1-ml volume of ethanol and fresh heparin plasma standards of DDC, Me-DDC (25–1500 nmol/l) and CS_2 (25–400 μmol/l) were spiked with 10 μl of internal standard (Prop-DDC) working solution to evaluate the necessity of using internal standards in combination with on-line precolumn purification HPLC assays. The study was made by comparing peak-area recovery from ethanol and plasma standards both with and without the addition of internal standard. All standard solutions were injected onto the precolumn and, for normal reversed-phase HPLC comparison, the ethanol standard solution was also directly injected onto the analytical column.

Protein binding

To evaluate the influence of plasma protein binding of the compounds purified, enriched and partitioned onto the precolumn, the peak-area recovery from ethanol and plasma standard solutions were compared. Standard solutions were injected onto both columns as described above.

In vitro studies

DSF was added to fresh heparin plasma in concentrations ranging from 3.4 to 3400 nmol/l to evaluate the stability and recovery of DSF in human plasma.

In vivo studies

The DDC and Me-DDC time courses were followed in five male alcoholics who had received a single oral 400-mg dose of DSF (Antabus). All patients had not had DSF treatment for at least three days before the present administration. Heparinized blood samples were drawn before and 1, 2, 4, 8, 16 and 24 h after medication and analysed immediately (within 5 min) for determination of unchanged DSF. The DDC metabolite was quantified as its ethyl ester derivative in the same analysis as for the more stable Me-DDC analogue, while $Cu(DDC)_2$ and CS_2 were analysed separately.

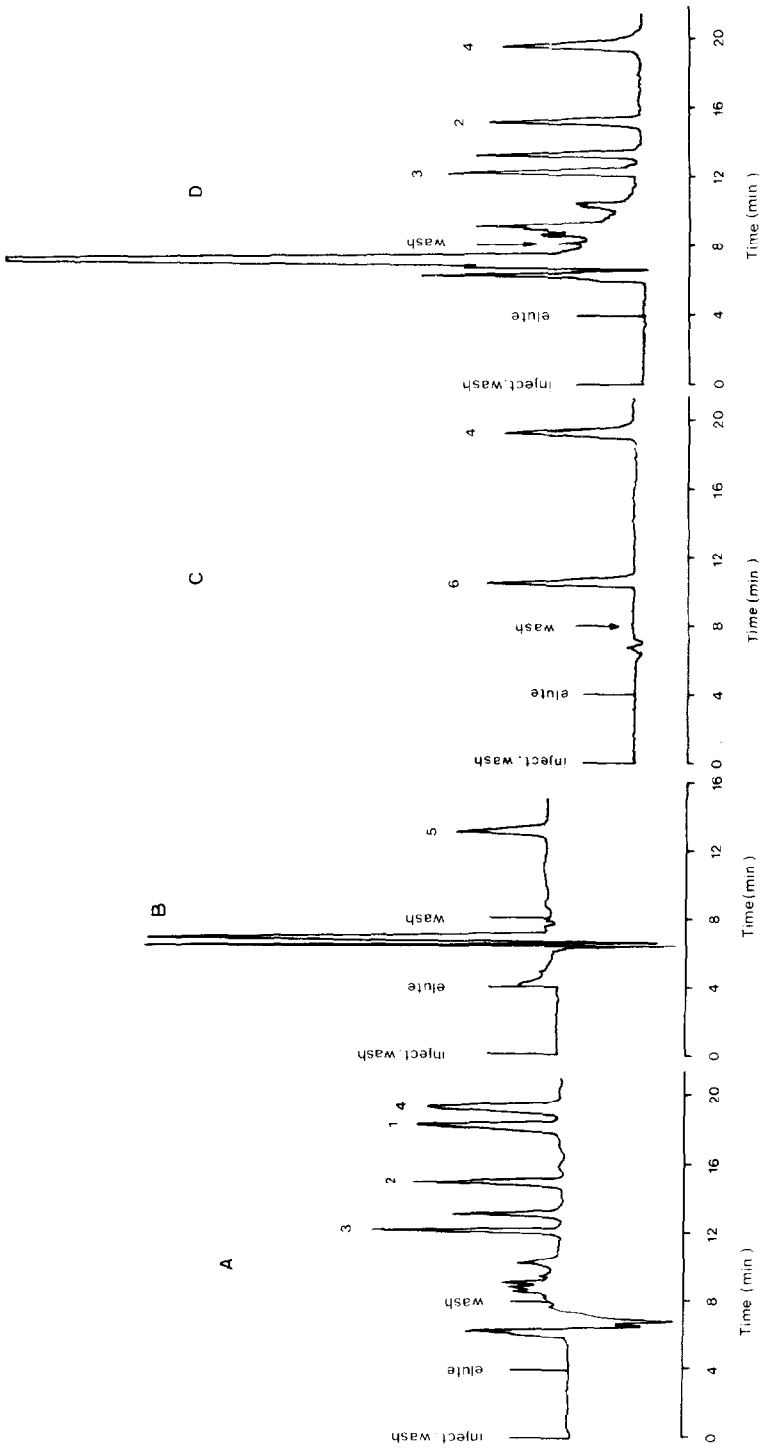
RESULTS AND DISCUSSION

The isocratic separations of DSF, DDC (Et-DDC), Me-DDC, CS_2 (DMDC), Prop-DDC (internal standard) and $Cu(DDC)_2$ in ethanol and heparin plasma standard working solutions are illustrated in Fig. 2A—F and a blank plasma in G, respectively. Sample on-line precolumn purification, enrichment and complete separation of substances of interest were obtained in 15 min.

When DSF was added to fresh heparin plasma in concentrations ranging from 3.4 to 3400 nmol/l, no detectable amounts of unchanged drug could be found (Fig. 2D) even though the samples were analysed within 5 min after sample preparation [5].

The retention times of DSF, DDC (Et-DDC), Me-DDC, CS_2 (DMDC), internal standard and $Cu(DDC)_2$ were in the two different elution profiles 18.3, 15.5, 12.5, 10.5, 19.5 and 13.0 min, respectively.

The recovery of the derivatized ethyl ester analogue of DDC previously reported at 78 ± 2.4% [3] was, in our study, found to be 102 ± 4.1%. To improve the conditions for optimal reaction yield, i.e. to completely reduce all DDC bound to plasma protein-bound cupric ions and to convert it to its ethyl ester analogue, it was found necessary to add 2-mercaptoethanol as a reducing agent to the reaction mixture. To determine CS_2 directly from plasma by HPLC without interference from other DSF metabolites, it was found necessary to form a dimethyldithiocarbamate ethyl ester derivative to CS_2. The CS_2 recovery from plasma was found to be 72 ± 3.5% after addition of the reducing agent, dithiothreitol. This value is approximately 24% higher than previously reported [3] and is probably due to a decreased CS_2 non-covalent interaction with plasma proteins. During chromatography, most of the unreacted ethyl iodide and reducing agents were eliminated in the waste during the precolumn washing cycle. To avoid undesired reduction of the parent drug and of the later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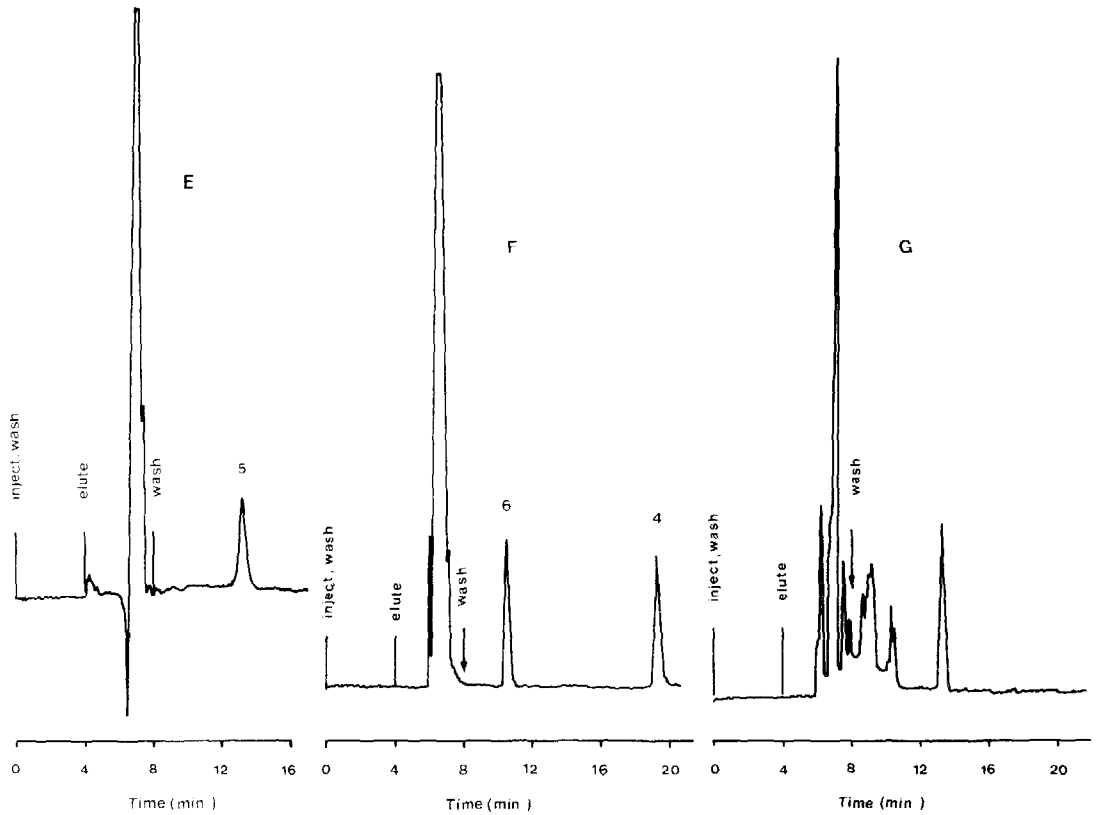

Fig. 2. HPLC using isocratic separations of an ethanol working standard solution (600 nmol/l) containing: (A) DSF (1), DDC (2), Me-DDC (3), Prop-DDC (4); (B) $Cu(DDC)_2$ (5); (C) CS_2 (6). (D, E, F) Plasma standard working solutions (600 nmol/l). (G) Blank plasma. The peak eluted after approximately 13 min is a water contamination enriched on the precolumn. Conditions: washing buffer 0.01 mol/l phosphate buffer (pH 7.5) and 0.005 mol/l EDTA; mobile phases 0.01 mol/l phosphate buffer (pH 7.5)—acetonitrile in ratios of 40:60 for DSF, DDC, Me-DDC, CS_2 and Prop-DDC and 15:85 for $Cu(DDC)_2$. Flow-rate 1.0 ml/min; injection volumes of 20 μl (CS_2), 100 μl (DSF, DDC, Me-DDC and CS_2) and 500 μl [$Cu(DDC)_2$].

observed metabolite, $Cu(DDC)_2$ [5], these compounds were analysed directly from plasma without the addition of alkylating reagents. Plasma standard graphs of DDC (Et-DDC), Me-DDC, $Cu(DDC)_2$ and CS_2 are linear over their concentration ranges, with correlation coefficients of 0.998–0.999. The detection limits are approximately 10 pmol/ml for DSF, 25 pmol/ml for DDC (Et-DDC) and CS_2 (DMDC), 20 pmol/ml for Me-DDC and 30 pmol/ml for $Cu(DDC)_2$.

The recoveries of Me-DDC and DDC given in Table I are almost complete when analysed without the addition of internal standard. With the addition of internal standard, the deviation was somewhat higher. The difference in CS_2 recovery, also confirmed in Table III (slopes 1.4), is probably due to interaction of CS_2 with proteins as discussed above.

As can be seen in Table III, the correlation relationships of DDC (Et-DDC), CS_2 and Me-DDC standard graphs from directly injected ethanol and plasma standard working solutions with and without the addition of internal standard are almost equivalent. The correlation coefficients are in the range

TABLE I

RECOVERY OF FIVE DETERMINATIONS AFTER ADDITION OF Me-DDC, DDC (100 AND 1000 nmol/l) AND CS_2 (50 AND 400 μmol/l) TO PLASMA AND ETHANOL, RESPECTIVELY

Metabolite	Recovery (mean ± S.D.) (%)			
	100 nmol/l	1000 nmol/l	50 μmol/l	400 μmol/l
Me-DDC*	99 ± 3	95 ± 2	—	—
DDC*	101 ± 7	100 ± 2	—	-
CS_2*	—	—	68 ± 7	71 ± 1
Me-DDC**	96 ± 5	108 ± 6	—	—
DDC**	91 ± 7	107 ± 2	—	—
CS_2**	—	—	74 ± 3	72 ± 1

*Without addition of internal standard.
**With addition of internal standard.

TABLE II

PRECISION FROM FIVE DETERMINATIONS AFTER ADDITION OF Me-DDC, DDC (100 AND 1000 nmol/l) AND CS_2 (50 AND 400 μmol/l) TO PLASMA

Values in parentheses are coefficients of variation expressed in %.

Metabolite	Concentration found (mean ± S.D.)			
	100 nmol/l	1000 nmol/l	50 μmol/l	400 μmol/l
Me-DDC*	101 ± 3 (3.0)	856 ± 20 (2.3)	—	—
DDC*	99 ± 7 (7.0)	964 ± 21 (2.2)	—	—
CS_2*	—	—	34 ± 3 (8.8)	286 ± 4 (1.4)
Me-DDC**	102 ± 5 (4.8)	1073 ± 60 (5.6)	—	—
DDC**	97 ± 8 (8.2)	1000 ± 18 (1.8)	—	—
CS_2**	—	—	40 ± 2 (5.0)	315 ± 5 (1.6)

*Without addition of internal standard.
**With addition of internal standard.

TABLE III

CORRELATION COEFFICIENTS AND CALIBRATION CURVE SLOPES FROM PEAK-AREA RECOVERY COMPARISONS AFTER INJECTION OF ETHANOL AND PLASMA STANDARD WORKING SOLUTIONS OF Me-DDC, DDC (25—1500 nmol/l) AND CS_2 (25—400 μmol/l)

All values are an average of three determinations. The values between parentheses are the calibration curve slopes.

Metabolite	Ethanol standard (directly injected) versus ethanol standard (injected onto precolumn)	Ethanol standard (directly injected) versus plasma standard (injected onto precolumn)	Ethanol standard (injected onto precolumn) versus plasma standard (injected onto precolumn)
Me-DDC*	0.999 (1.0)	0.998 (1.1)	0.999 (1.1)
DDC*	0.998 (1.0)	0.998 (1.1)	0.998 (1.1)
CS_2*	0.999 (0.96)	0.998 (1.4)	0.998 (1.4)
Me-DDC**	—	0.994 (1.0)	—
DDC**	—	0.989 (1.0)	—
CS_2**	0.998 (1.0)	0.998 (1.4)	0.993 (1.3)

*Without addition of internal standard.
**With addition of internal standard.

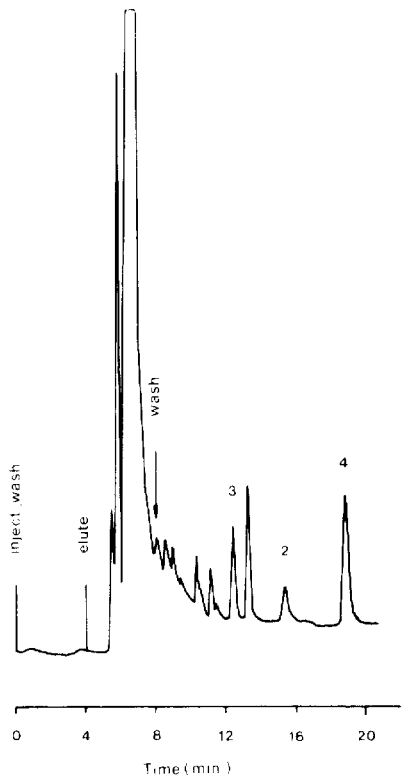

Fig. 3. HPLC profile using an isocratic separation of a patient plasma sample containing 240 nmol/l DDC and 515 nmol/l Me-DDC. Conditions and peak numbers as in Fig. 2.

0.989—0.999 (slopes 0.96—1.15). When using reversed-phase precolumn purification and enrichment of the hydrophobic metabolites of DSF, there is obviously no need for an additional internal standard.

The precision of the method tested at two plasma concentration levels is illustrated in Table II. The coefficients of variation obtained are as expected, higher at the lower plasma concentration levels.

The correlation coefficients in Table III and the recoveries in Table I also show that besides extraction and isolation, all the metabolites, except CS_2, are quantitatively partitioned to give a complete retardation on the precolumn without any influence from plasma protein binding [6].

These results suggest that the hydrophobic metabolites, including the even more lipophilic $Cu(DDC)_2$, could be completely and quantitatively analysed directly from plasma without use of an internal standard and without loss of analysed material as in a conventional sample extraction and preparation procedure.

Fig. 3 shows a heparin plasma sample from a male alcoholic drawn 4 h after an oral 400-mg dose of DSF. The plasma concentrations of DDC and Me-DDC

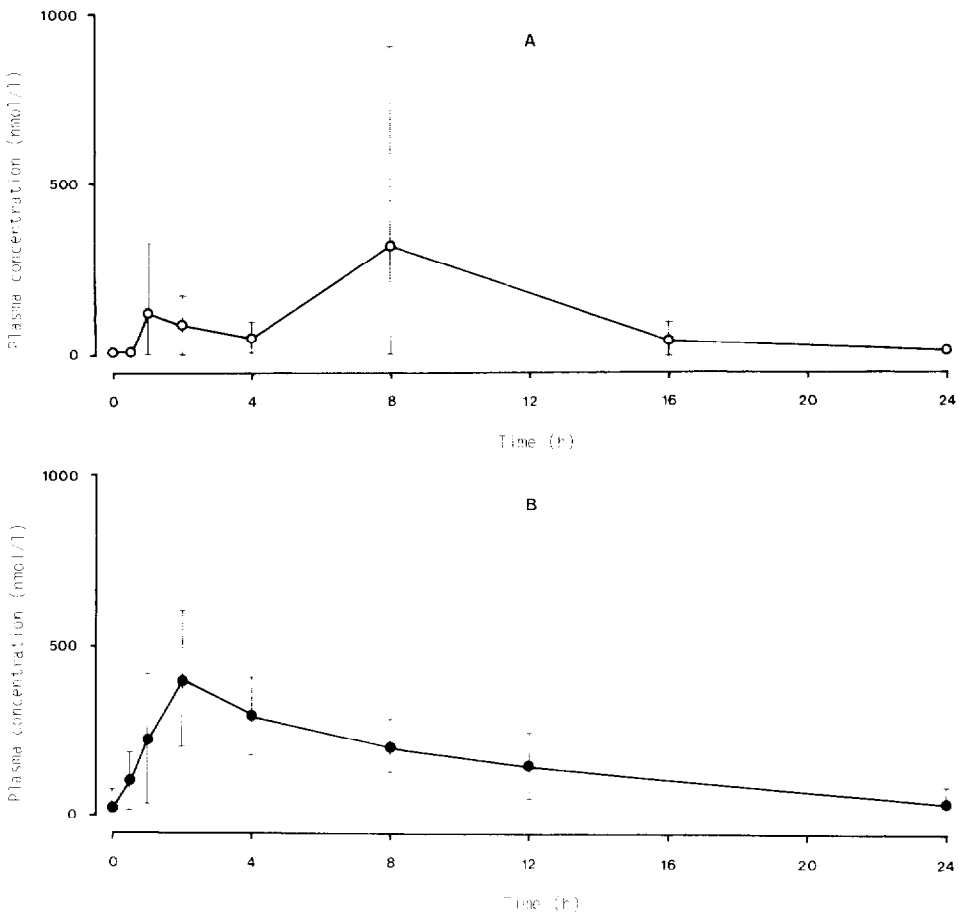

Fig. 4. DDC (A) and Me-DDC (B) plasma—time courses (± S.D.) after a single oral 400-mg dose of DSF administered to five alcoholics.

were 240 and 515 nmol/l, respectively, while DSF was below and $Cu(DDC)_2$ and CS_2 were just above the detection limit of the HPLC assay used. In two out of four patients, detectable amounts of the hydrophobic metabolite, $Cu(DDC)_2$, were found in plasma from 0.5 h to 8 h after DSF administration [5]. The observation that DSF is unstable in human blood and plasma [3–5] was confirmed in our study. No detectable plasma concentrations of unchanged drug were found in any of the five patients examined. Preliminary results suggest that the reason for the very low plasma CS_2 levels is that the volatile CS_2 metabolite is evaporated from the blood in the vacuumized tubes during blood collection.

The mean plasma concentration–time profiles of DDC and Me-DDC in five alcoholics are shown in Fig. 4.

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