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SIMULTANEOUS DETERMINATION OF DISULFIRAM AND TWO OF ITS DITHIOCARBAMATE METABOLITES IN HUMAN PLASMA BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple, sensitive, reversed-phase liquid chromatographic assay is reported for the simultaneous determination of disulfiram, diethyldithiocarbamate (DDC) and its methyl ester (MeDDC) in human plasma. A single-step extractive ethylation converts DDC to its ethyl ester which is then separated from endogenously produced MeDDC and parent disulfiram on an alkylphenyl column. The method is sufficiently sensitive (25 ng/ml) to permit DDC and MeDDC determinations in patients receiving therapeutic doses of disulfiram.

INTRODUCTION

Disulfiram (tetraethylthiuram disulfide, Antabuse®) (DSF) is widely used clinically in the treatment of alcoholism. The drug renders the patient sensitive to ethyl alcohol although the underlying mechanism by which the drug—ethanol reaction (DER) occurs is still poorly understood. Little is known about the pharmacokinetics of DSF and its metabolites in man. It also remains to be determined whether relationships exist between the plasma levels of parent drug or metabolites and the intensity of the DER induced by the drug. Toxic side effects such as neuropathy have also not been related clearly to the drug's disposition. Such relationships might serve as the basis for the rational design of long term disulfiram delivery systems. A major obstacle to resolving these questions has been the lack of availability of sensitive analytical methods for the determination of DSF and its major metabolites (Fig. 1) in human plasma.

While spectrophotometric, polarographic and gas chromatographic methods have been reported [1—6] a liquid chromatographic technique would seem more appropriate because of its potential convenience, selectivity, sensitivity

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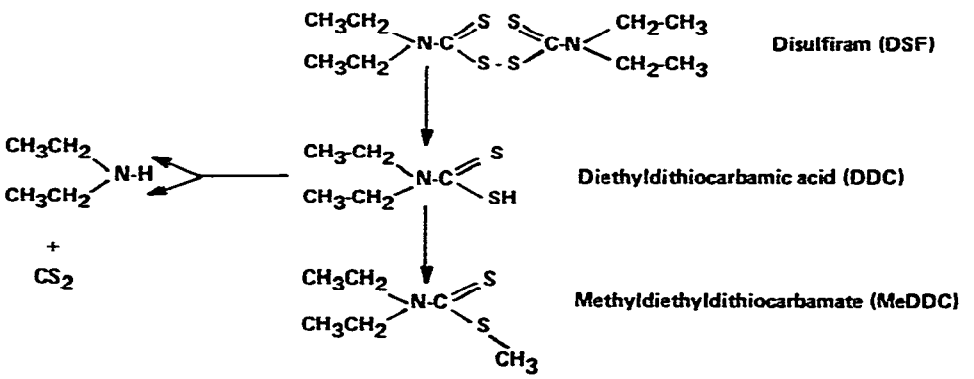

Fig. 1. Pathways for metabolism of disulfiram to its first degradation products in man.

and suitability to the quantitation of labile drugs and metabolites. Jensen and Faiman [7] and Pedersen [8] have determined DSF and its endogenously-produced metabolite methyldiethyldithiocarbamate (MeDDC) by normal-phase high-performance liquid chromatography (HPLC). They utilized extractive methylation of diethyldithiocarbamate (DDC) to determine this metabolite after removing and quantitating endogenously-produced MeDDC. Our objective was to develop a simple reversed-phase chromatographic technique that would quantitate both metabolites simultaneously rather than through two different extraction procedures.

EXPERIMENTAL

Chemicals

DSF was a gift of Ayerst Labs. (New York, NY, U.S.A.). DDC was purchased as the sodium salt from Sigma (St. Louis, MO, U.S.A.) and used without further purification. Methyl, ethyl and propyl esters of DDC were synthesized in our laboratory (see below). Methyl iodide and ethyl iodide (Aldrich, Milwaukee, WI, U.S.A.) were distilled from glass before use. Organic solvents were distilled in glass (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). All other chemicals were reagent grade and used without further purification. Water was deionized and double distilled in glass.

Apparatus

A Model 5000 gradient liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) and a Model 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) each equipped with a Model 440 absorbance detector (280 nm), a Phenyl/Corasil Bondapak precolumn (5 cm × 3.9 mm I.D. and a 10-μm alkylphenyl μBondapak column (30 cm × 3.9 mm I.D.) (Waters Assoc.) were used for gradient and isocratic separations, respectively. Mobile phase was a mixture of acetonitrile—water (52:48) for isocratic separations and was programmed to increase from 51:49 to 75:25 over 10 min for gradient separations. Mobile phase flow-rate was maintained at 1.5 ml/min and column temperature at 37°C.

Substitution of 0.01 *M* acetate buffer (pH 4) for the water did not affect the chromatography.

Synthesis of diethyldithiocarbamate esters

Approximately 0.025 mole of alkyl halide were added with constant stirring to an equimolar quantity of sodium DDC dissolved in approximately 7 ml of absolute ethanol. After 20—30 min reaction at room temperature an equal volume of water was added. The lower yellow oily layer was separated, dried over calcium chloride, filtered and vacuum distilled (Table I). Infrared and NMR spectroscopy were used to confirm the identity of the product.

TABLE I

PARAMETERS FOR DIETHYLDITHIOCARBAMATE ESTER SYNTHESIS

Alkyl halide	Ester	Distillation temperature (°C)	Distillation pressure (mm Hg)
Methyl iodide	MeDDC	89	1.5
Ethyl iodide	EtDDC	112	2.6—4.0
n-Propyl bromide	PrDDC	99	0.6

Preparation of standards

Stock solutions of DDC esters, sodium DDC and DSF (about 1 mg/ml) were prepared by dissolving the pure compound in acetonitrile. Dilutions of 1:50 and 1:100 of these stocks provided working solutions for spiking of plasma samples. Stocks of the esters were stable at 4°C for several months, but sodium DDC and DSF had to be freshly prepared prior to use.

Calibration standards containing between 50 and 500 ng/ml were prepared by addition of an appropriate quantity of working stock solution to drug-free plasma using *n*-propyl-DDC as the internal standard. Standards and samples were extracted as described below.

Extraction procedure

A 2-ml aliquot of plasma was placed in a 16 × 125 mm test tube containing an equal volume of 0.05 *M* Tris buffer (pH 8.5) to which has been added EDTA (0.01 *M*). Ethyl iodide (20 μl) was added and, following 30 sec of vortex mixing, the mixture was incubated at 40°C for 30 min and cooled to room temperature. Zinc sulfate (500 mg) and diethyl ether (4 ml) were added and the tubes gently inverted by hand for 3 min. After centrifugation for 6 min at 1200 *g*, the ether layer was removed and placed in a clean test tube. Nine milliliters of carbonate buffer (pH 11, 0.1 *M*) were added followed by 3 min of inversion and 6 min of centrifugation. The carbonate-washed ether layer was transferred to a clean tube, concentrated to 100 μl under nitrogen, diluted to 1 ml with acetonitrile and reconcentrated to about 200 μl. A 15—20 μl aliquot was injected onto the column for chromatographic analysis. Fig. 2 provides a schematic summary of the procedure.

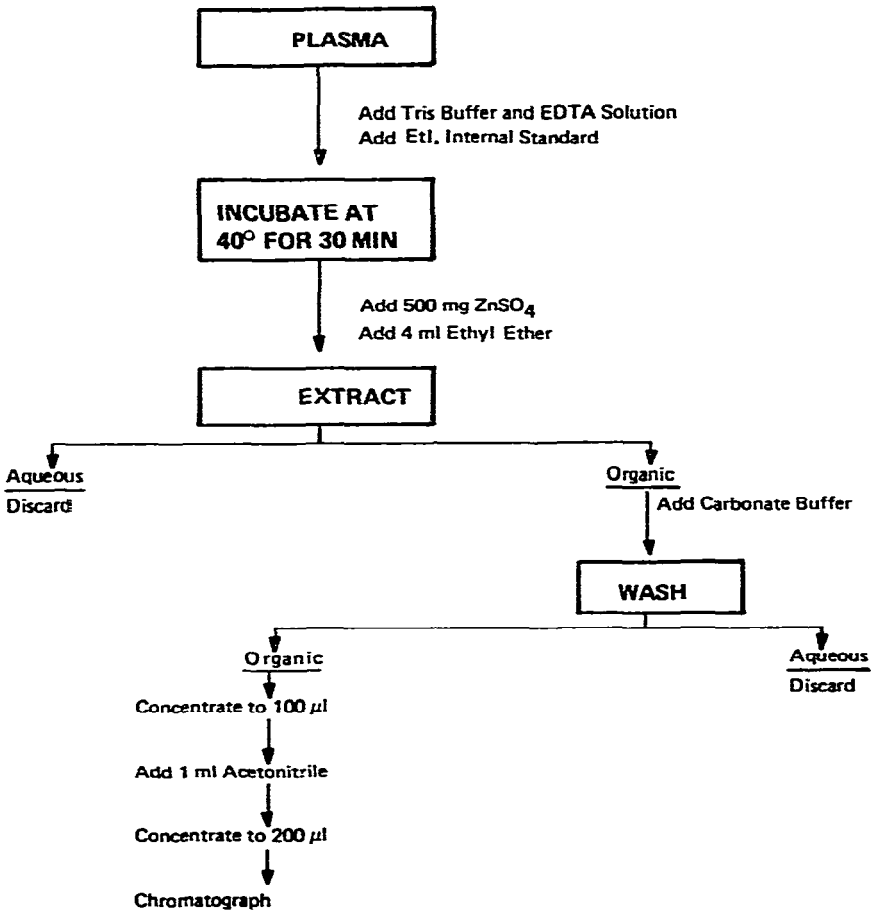

Fig. 2. Schematic of plasma sample workup for disulfiram and two of its diethyldithiocarbamate metabolites.

Standard curves

Standard curves were prepared by adding known quantities of DDC or MeDDC to a fixed concentration (400 ng/ml) of propyl-DDC in drug-free plasma. Samples were analyzed as described above and peak height ratios of drug to internal standard were plotted against drug concentration. Peak height ratios of unknown samples were similarly determined and concentrations calculated from the standard curve.

Recovery

Recoveries of the methyl and ethyl esters of DDC were determined by comparison of peak height ratios determined for an extracted plasma sample with those obtained for an unextracted standard in acetonitrile. In each case propyl-DDC was added as an injection volume standard just prior to chromatography. Efficiency of the ethylation reaction was also determined by comparing samples of DDC which were extractively ethylated with those containing equimolar amounts of the pure ethyl ester.

Human study

Three male alcoholic volunteers were utilized. Each was hospitalized on a daily regimen of 250 mg DSF as a single oral dose. Two of these were given their normal dose of drug at 8 a.m. and plasma samples were drawn 2 h post dosing. At this time breath tests using McKees reagent [9] were positive for carbon disulfide. A third subject was given a 500-mg dose of DSF and similarly sampled. Plasma was separated and analyzed immediately. Plasma aliquots were also maintained at room temperature for 2 h and reassayed to determine the in vitro stability of the analytes.

RESULTS AND DISCUSSION

Fig. 3a and b shows chromatograms depicting the isocratic separation of the methyl, ethyl and propyl esters of DDC and DSF from a neat mixture of the components in acetonitrile and an extracted plasma sample, respectively. DSF degraded rapidly upon addition to plasma and at the low concentrations (less than 500 ng/ml) used was in evidence only as a less than quantitative amount of DDC when the plasma was subsequently analyzed. Fig. 4 depicts the results of our gradient separation of components from acetonitrile solution. The only apparent chromatographic advantage over isocratic separation is a somewhat sharper and taller disulfiram peak for this lipophilic and highly retained material. Calibration curves for DDC and its methyl ester from plasma over the 50—500 ng/ml concentration range were linear (r = 0.999). Coefficients of variation (within-run) were 4.3% and 6.1% for DDC and MeDDC, respectively, at 100 ng/ml (n = 6) and 3.8% and 6.0% at 400 ng/ml (n = 6). DDC and MeDDC could be accurately quantitated in plasma down to a concentration of 25 ng/ml, and each appeared to be independent of concentration within the range studied.

Percentage conversion of DDC to its ethyl ester under the derivatization conditions invoked in our procedure was 78 ± 3.4% (mean ± S.D., n = 6). Recoveries of the ethyl-DDC formed and of MeDDC were 67 ± 6.2% (n = 6) and 68 ± 3.5% (n = 6), respectively. Both conversion and recovery were independent of concentration over the concentration range studied.

Several variables in the extractive alkylation procedure were optimized in the course of developing the procedure. The conversion of DDC to its ethyl ester was independent of the amount of ethyl iodide added above 5 μl/ml plasma and independent of reaction time beyond 20 min. The reaction temperature of 40°C was deemed adequate to provide an increased rate of reaction and solubility of ethyl iodide above that obtained at room temperature with minimal exposure of the other components of the system to heat. Zinc sulfate was selected as a plasma protein precipitant because it provided extremely clean plasma extracts. While chlorinated hydrocarbons such as methylene chloride and chloroform work well as the extraction solvent, they are inconvenient because they tend to form gels on shaking and generally sequester the drug in a difficult to separate lower phase. It must be noted that the esters of DDC were found to be sufficiently volatile to render evaporation to dryness inappropriate. We found the small quantities of residual diethyl ether present in the injected sample to have no effect on the chromatography. Only very small amounts of

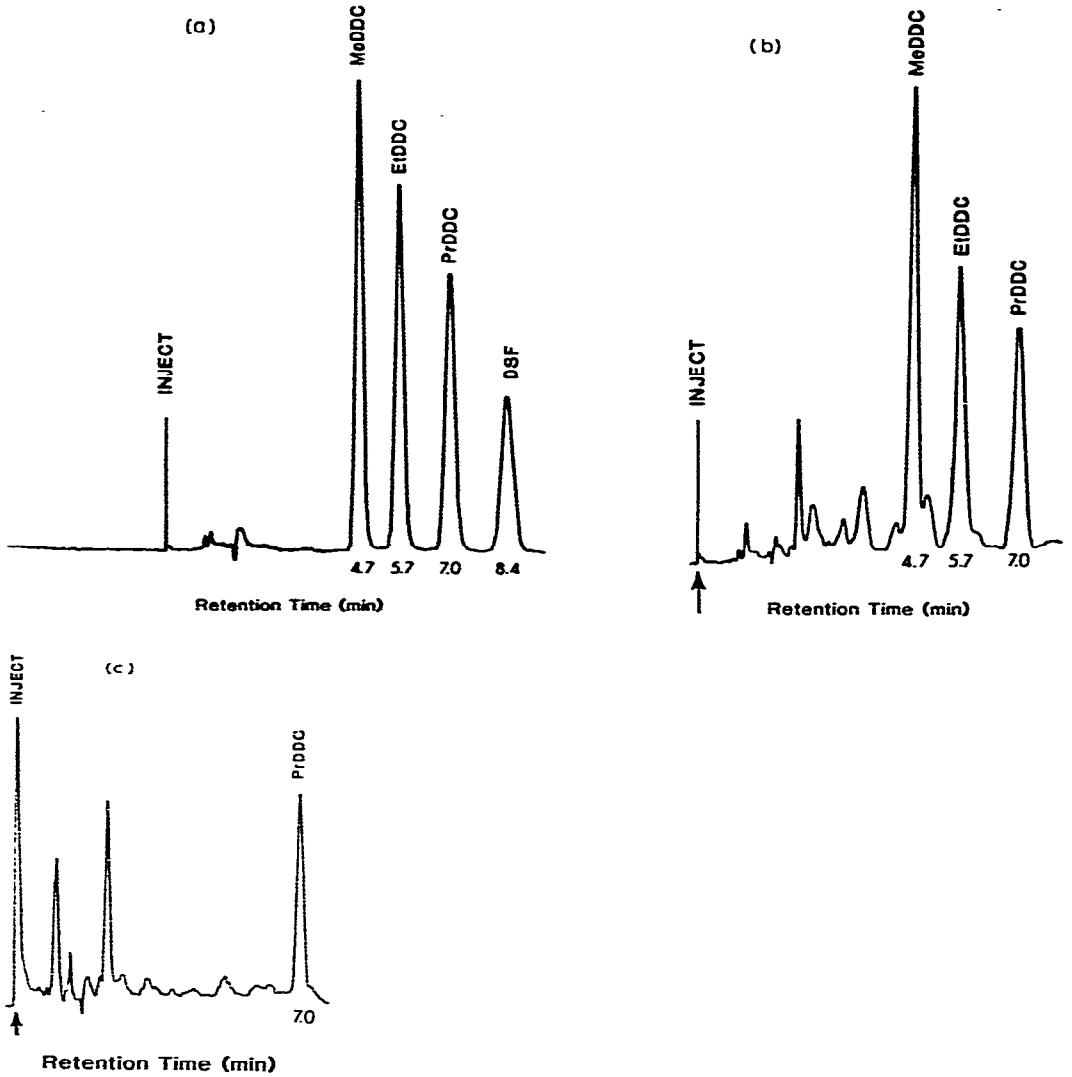

Fig. 3. Isocratic separation of disulfiram (DSF) and two of its diethyldithiocarbamate metabolites on a μBondapak alkylphenyl HPLC column. (a) Neat mixture of components in 52% acetonitrile in distilled water. Conditions: injection volume 20 μl; mass of each of the esters 27.2 ng/20 μl; mass of DSF 40 ng/20 μl. (b) Components extracted from human plasma at 200 ng/ml. (c) Blank plasma. Attenuation 0.01 a.u.f.s.

unreacted ethyl iodide were found in the sample applied to the column when diethyl ether was used whereas a large ethyl iodide peak eluting just prior to MeDDC was evident when methylene chloride or chloroform was substituted.

Fig. 5 shows a chromatographic analysis of the plasma from a male alcoholic drawn 2 h after a 500-mg oral dose of DSF. Plasma levels of MeDDC and DDC were 49 and 43 ng/ml, respectively. Reanalysis of the above sample after 2 h at 25°C resulted in an 8% decrease in the level of DDC while MeDDC remained

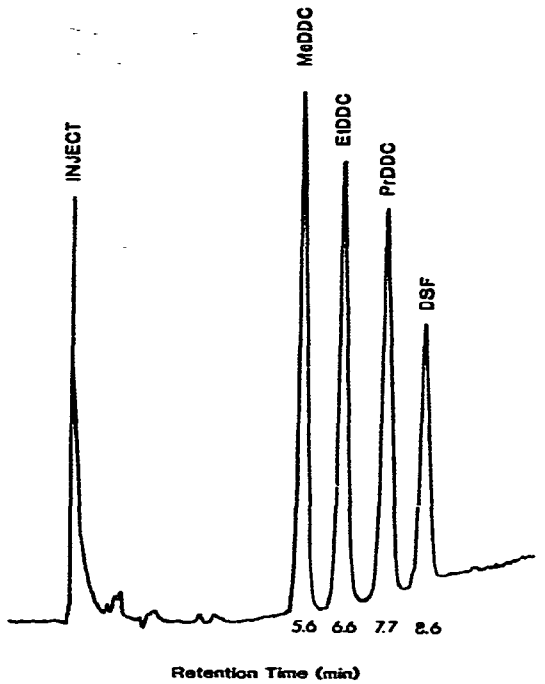

Fig. 4. Gradient separation of disulfiram (DSF) and two of its diethyldithiocarbamate metabolites on a μBondapak alkylphenyl HPLC column. Conditions: gradient from 51% acetonitrile in distilled water at 0 min to 75% acetonitrile in distilled water at 10 min; injection volume 20 μl; mass of each of the esters 27.2 ng/20 μl; mass of DSF 40 ng/20 μl; attenuation 0.01 a.u.f.s.

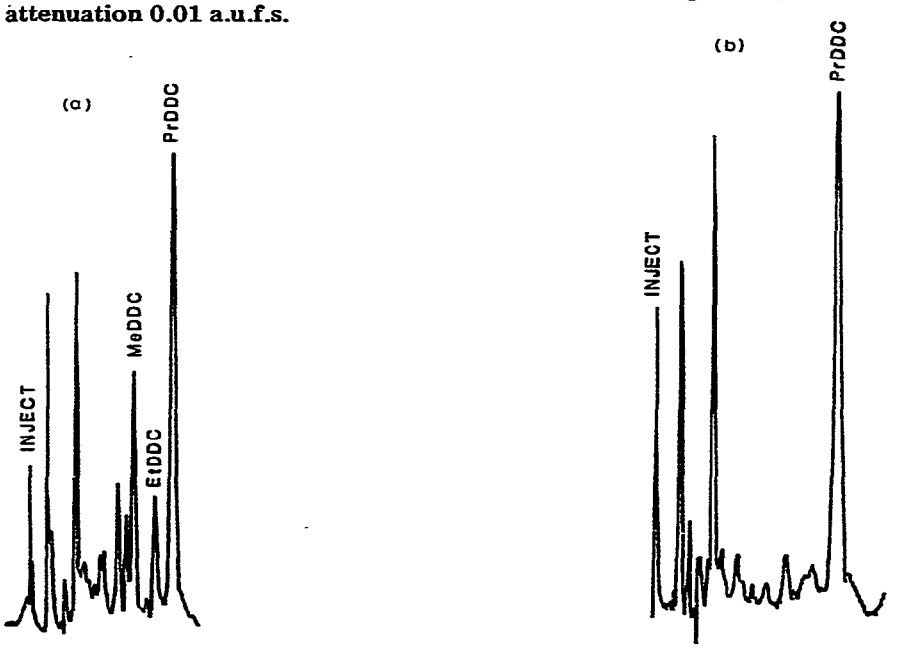

Fig. 5. Chromatograms of (a) plasma extract 2 h following administration of a 500-mg oral dose of disulfiram to a male alcoholic; MeDDC = 49 ng/ml, DDC = 43 ng/ml; (b) blank plasma. Attenuation 0.005 a.u.f.s.

unaffected. Two other patients who had been taking 250-mg daily doses of DSF did not achieve quantitable levels of DDC, MeDDC or DSF 2 h following their morning dose of DSF.

Our observations on the instability of DSF in plasma seem to confirm those of Pedersen [8]. He found that the recovery of DSF from plasma was a function of concentration and was able to recover only 5% of added drug at the 500 ng/ml level. Our results showed the compound to be essentially unrecoverable below 500 ng/ml. Pedersen also found negligible quantities of parent DSF and very low levels (generally less than 75 ng/ml) of DDC and MeDDC in a volunteer given an 800-mg oral dose of DSF. The levels reported are in good accord with those found in the present study following a 500-mg oral dose. Since Pedersen found only small quantities of protein-disulfide bound drug in plasma, it is apparent that DDC and its methyl ester in plasma account for only a very small percentage of absorbed DSF during times when the drug is exerting its maximum pharmacological effects. Their apparently rapidly disappearance from the blood stream [8] relative to the duration of action of DSF also suggests that if they are central to the drug's action they may be acting irreversibly.

The assay developed here is easy to perform, precise and sufficiently sensitive to permit determination of DDC and MeDDC levels following therapeutic doses of DSF. The sensitivity might well be increased still further if additional cleanup of the plasma extracts could be achieved. This has not been accomplished because of the combined lipophilicity of the compounds being analyzed and their lack of an ionizable group which might permit their back-extraction into aqueous solvents. The single-step extractive alkylation offers the added advantage of permitting simultaneous determination of DDC and its methyl ester on a reversed-phase system, obviating the need for prior removal of methyl ester.

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REFERENCES

1 G. Domar, A. Eredga and H. Linderholm, Acta Chem. Scand., 3 (1949) 1441.
2 R. Fried, A.N. Masoud and M. Francis, J. Pharm. Sci., 62 (1973) 1368.
3 D.G. Prue, C.R. Warner and B.T. Kho, J. Pharm. Sci., 61 (1972) 249.
4 E.C. Gregg and W.P. Tyler, J. Amer. Chem. Soc., 72 (1950) 4561.
5 J. Wells and E. Koves, J. Chromatogr., 92 (1974) 442.
6 J. Cobby, M. Mayerson and S. Selliah, J. Pharmacol. Exp. Ther., 202 (1977) 724.
7 J.C. Jensen and M.D. Faiman, J. Chromatogr., 181 (1980) 407.
8 S.B. Pedersen, Arch. Pharm. Chem. Sci. Ed., 8 (1980) 65.
9 W.K. Rogers, M. Wilson and C.E. Becker, Alcoholism: Clin. Exp. Res., 2 (1978) 375.