Journal of Chromatography, 181 (1980) 407—416
Biomedical Applications

CHROMBIO. 464

DETERMINATION OF DISULFIRAM AND METABOLITES FROM BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received July 11th, 1979; revised manuscript received October 17th, 1979)

SUMMARY

A high-performance liquid chromatographic method is described for the determination of disulfiram, diethyldithiocarbamate, diethyldithiocarbamate methyl ester, carbon disulfide, and diethylamine from a single sample of plasma or urine. The analytical procedure is based on a quantitative stepwise extraction of disulfiram and diethyldithiocarbamate methyl ester, or the conversion of diethyldithiocarbamic acid, carbon disulfide and diethylamine to diethyldithiocarbamate methyl ester for chromatographical determination. The procedure is specific, precise and simple. The application of the analytical methods developed for the determination of disulfiram and the various metabolites in plasma from mice given disulfiram intraperitoneally or humans given Antabuse orally is illustrated.

INTRODUCTION

Disulfiram (tetraethylthiuram disulfide, Antabuse®) (DSF) has been used in the treatment of alcoholism since its introduction by Hald et al. [1]. Even though DSF has been used in alcoholics for approximately thirty years, little is known about its absorption and elimination characteristics. The main reason for this paucity of information appears to be due to the lack of an analytical method suitable for use in humans which can determine DSF and its metabolites in biological fluids.

A number of different analytical methods for the determination of disulfiram and its metabolites have been employed. These include spectrophotometry [2—8], polarography [9—13], proton magnetic resonance [14] and gas chromatography [15—17]. The use of radioactive disulfiram in man and animals also has been employed to study the metabolites and excretion characteristics of DSF [18—21]. Although the various methods employed are suitable, none of these appear to combine the criteria of convenience, speed, and with some of the methods, sensitivity. Furthermore, the use of radioactive DSF

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limits the usefulness of this method in studies of a patient population. The studies to be reported here describe a high-performance liquid chromatographic (HPLC) method which appears to fulfil many of the desired criteria.

EXPERIMENTAL

Chemicals

DSF was supplied by Ayerst Laboratories (New York., U.S.A.). Diethyldithiocarbamate (DDTC) was purchased from Sigma (St. Louis, Mo., U.S.A.), and diethyldithiocarbamate methyl ester (DDTC-Me) was prepared in our laboratory [21]. All solvents were of analytical grade and used without further purification. The heptane must be of fluorescence grade. Solvents were tested for purity by evaporating aliquots equal in volume to those used in the extraction procedure, and chromatographing them in the liquid chromatograph at the highest sensitivity.

Apparatus

A Perkin-Elmer (Norwalk, Conn., U.S.A.) Series 2 high-performance liquid chromatograph equipped with a 10 μm silica A column (25 cm × 0.26 cm) and a Model 440 absorbance detector at 254 nm (Waters Assoc., Milford, Mass., U.S.A.) were used in these studies. (Note: more recently a 5 μm, 25 cm Spherisorb silica column from Laboratory Data Control (Riviera Beach, Fla., U.S.A.) was used which improved peak sharpness and retention times.) The mobile phase employed was a mixture of heptane—tetrahydrofuran—methanol (97.6:2.2:0.2) with a flow-rate of 1.2 ml/min.

Preparation of standards

The internal standard employed was ethyl-*p*-nitrobenzoate. The internal standard solution was prepared by adding ethyl-*p*-nitrobenzoate to analytical grade ethanol, and the mixture then added to chloroform to make a final concentration of either 0.2 μg/ml or 1 μg/ml depending upon need.

Stock solutions of DSF, DDTC, DDTC-Me, diethylamine (DEA) and carbon disulfide (CS_2) in methanol were prepared at concentrations of 1 mg/ml. Dilutions when necessary were made to bring the final volume added to the sample to less than or equal to 20 μl/ml. Standards added to plasma, urine, or buffer were in a range from 0.5 to 2 μg/ml. The standards were extracted as described below.

Extraction procedure

The extraction solution used was 0.01 *M* EDTA in 1.0% sodium chloride, adjusted to pH 8.5 by the addition of sodium hydroxide. Into a 15-ml screw-top tube, 1 ml of either plasma, urine or buffer, 2 ml of 0.01 *M* EDTA solution, and 5 ml of the chloroform solution containing the internal standard were added. The tubes were sealed and shaken on a Labquake Shaker (Lab Industries, Berkeley, Calif., U.S.A.) for 10 min, after which they were centrifuged at 1765 *g* (Dynac Centrifuge, Model 0101) for 10 min. Because there exists a potential for acid-catalyzed decomposition of DDTC, urine cannot be collected and stored. Therefore, 0.5 ml of 0.5 *N* sodium hydroxide must be

added to the urine sample. A 2.5-ml aliquot of the aqueous layer containing the DDTC, DEA and CS_2 was transferred to a second 15-ml screw-top tube. The chloroform from the organic phase was evaporated at room temperature under a stream of nitrogen to a final volume of approximately 50 μl. This was then injected into the liquid chromatograph and the concentration of DSF and DDTC-Me determined by comparison of their peak heights to those obtained using standard solutions. To the 2.5 ml aqueous phase, 150 μl of methyl iodide were added to convert the DDTC present to DDTC-Me. This mixture was then vortexed for 30 sec and left to stand at room temperature for 15 min, after which 5 ml of the chloroform extraction solution containing the internal standard were added. The tube was shaken for 10 min, and then centrifuged for 10 min at 1765 *g*.

The upper aqueous phase from the mixture was now separated and 1-ml aliquots placed into two 15-ml screw-top tubes (tubes A and B). The organic phase from this mixture was removed, placed in a conical centrifuge tube, and the chloroform evaporated to a final volume of 50 μl. This volume was injected into the chromatograph and the concentration of DDTC-Me determined. To tube A, 100 μl of CS_2 were added and left to stand for 10 min. This converted the DEA to DDTC. After 10 min, 100 μl of methyl iodide were added to methylate the DDTC. To tube B, 100 μl of DEA were added which converted the CS_2 to DDTC, and left to stand for 10 min; then 100 μl of methyl iodide were added. After the addition of the methyl iodide, both tubes A and B were stoppered, vortexed for 30 sec, and left to stand at room temperature for 15 min. This completed the methylation of DDTC to methyl ester.

At the end of the 15-min period, 5 ml of the chloroform extraction solution containing the internal standard were added to both tubes A and B, the tubes shaken for 10 min, and then centrifuged at 1765 *g* for 10 min. The chloroform layer from each tube was pipetted into two separate glass conical centrifuge tubes, and the chloroform evaporated under nitrogen at room temperature to a final volume of 50 μl. At this time, 10 μl of the final 50-μl volume was removed from each glass conical centrifuge tube, injected into the chromatograph, and DDTC-Me from each tube determined. The DDTC-Me peak height obtained was compared to the standard curve and represented the amount of DEA and CS_2 extracted from tubes A and B, respectively.

Standard curves

Standard curves were prepared by adding known amounts of DSF, DDTC-Me, DDTC, CS_2 and DEA to buffer, plasma or urine. Analysis for each was then carried out as described. The peak height ratios of DSF and DSF-Me to the internal standard were calculated and plotted against known concentrations. The peak height ratios of unknown samples were calculated in the same manner and compared to the standard curves.

Recovery

Recovery studies were carried out by the separate addition of 0.5 μg of DSF, DDTC, DDTC-Me or DEA and 5 μl of CS_2 to 1 ml of either physiological phosphate buffer, plasma or urine. DSF and the various metabolites were then extracted as described and the percentage recovery determined by comparison with a standard concentration of drug entity.

In vivo studies

Mice. Male mice (HA/ICR, Sprague—Dawley, Madison, Wisc., U.S.A.) weighing 30—35 g were given DSF 200 mg/kg intraperitoneally (i.p.). The DSF was solubilized with polysorbate 80, and then 1% methylcellulose was added to give a final preparation for injection. A volume of 0.1 ml of DSF suspension per 10 g of animal weight (200 mg/kg) was administered i.p. Mice were sacrificed by decapitation at 5, 10, 20 and 30 min after DSF administration. Blood was collected, and 50 μl of 1 *M* sodium citrate solution were added to prevent coagulation. After centrifugation at 1765 *g* for 10 min, 100—500 μl of plasma were taken and extracted as described above.

Human. Male alcoholic volunteers were given 500 mg of Antabuse at 8:00 a.m. after an overnight fast. An indwelling venous catheter was placed in the subject's arm and 4-ml blood samples were drawn into vacutainer tubes at various times after dosing. Plasma samples were then analyzed for DSF and the various metabolites.

RESULTS

A scheme illustrating the various steps in the extraction procedure is outlined in Fig. 1. In Fig. 2, a typical separation of DSF, DDTC-Me and internal standard in the mobile phase extracted from plasma and urine is shown. Fig. 2 also includes chromatograms for both plasma and urine blanks extracted in the same manner as plasma and urine containing DSF and DDTC-Me. No interference by the plasma and urine peaks with those of DSF and DDTC-Me was found. The solvent peaks shown in the blank samples are due to the increased sensitivity used for these injections. The retention times for DSF, DDTC-Me and the internal standard were 7.4, 3.1 and 4.3 min, respectively, for the mobile phase and flow-rate used.

The standard curve obtained for DSF and DDTC-Me in plasma is illustrated

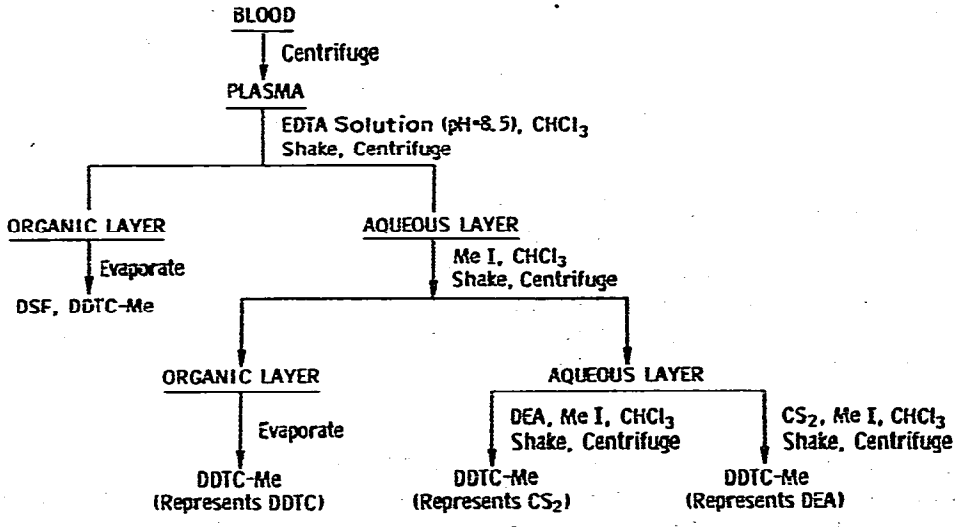

Fig. 1. Extraction scheme for disulfiram and metabolites. For extraction from urine or buffer, begin at plasma step.

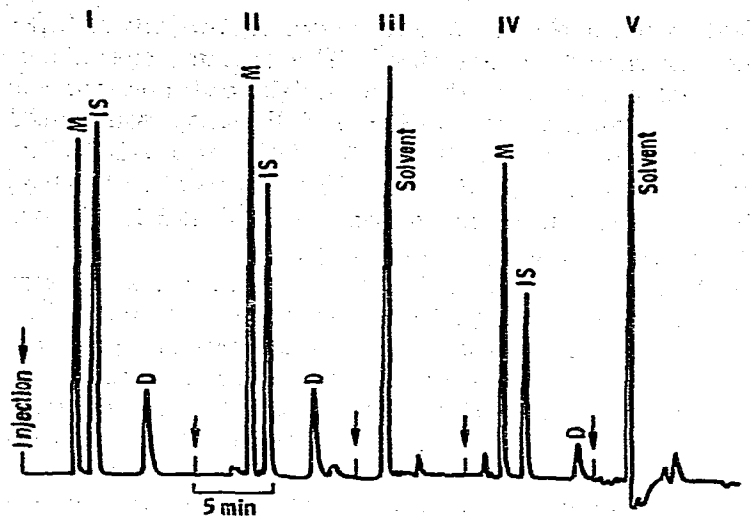

Fig. 2. HPLC chromatograms of DSF (D), DDTC-Me (M) and internal standard (IS) in mobile phase (I), plasma (II), and urine (IV). Plasma blank (III) and urine blank (V) are also shown. Points of injection are indicated by arrows. See text for column description, eluent, flow-rate and retention times.

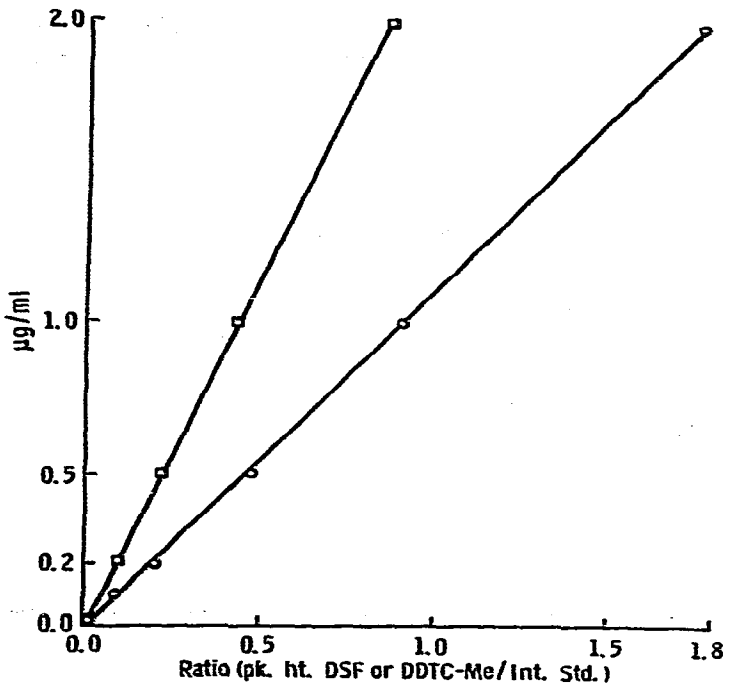

Fig. 3. Standard curves prepared by plotting concentration of DSF (□) or DDTC-Me (○) extracted from plasma against the peak height ratio of DSF or DDTC-Me/internal standard.

in Fig. 3. DSF and DDTC-Me were added to plasma in concentrations of 0.2—2.0 μg/ml and the samples extracted as described. The amount (μg/ml) of drug added was plotted against the ratio of the peak height obtained for the drug to the peak height of the internal standard. Good linearity was found with correlation coefficients of 0.999 and 0.995 for DSF and DDTC-Me, respectively. Standard curves for DDTC, CS_2 and DEA showed correlation coefficients of 0.995 or better. The minimum amount of DSF and DDTC-Me that could be detected was 5.0 ng and 2.5 ng, respectively.

The recoveries of DSF, DDTC, DDTC-Me, DEA and CS_2 from buffer, plasma and urine are given in Table I. Almost complete recovery was obtained when DSF and the various metabolites were added to phosphate buffer. When added to either plasma or urine, however, recovery of DSF and metabolites was not complete. Recoveries for DDTC and DDTC-Me from plasma and urine were reasonably good, even though only 71% of added DDTC could be recovered from urine. The values obtained are the mean of ten determinations, except for the DDTC study which is the mean of nine determinations. The percentage recovery was not influenced by changes in the concentration of DSF or the various metabolites in the concentration range studied. This is suggested from the good linearity of the standard curves shown in Fig. 3. Various experiments during the course of these studies verified this result.

The accuracy and reproducibility of the analytical method developed is illustrated in Table II. After the addition of 0.5 μg/ml of DSF, DDTC, DDTC-Me, DEA and 5.0 μg/ml of CS_2 to plasma, the amount added compared favor-

TABLE I

PERCENTAGE RECOVERY

Values are the average percentage recovery of ten determinations ± S.E. except for the DDTC samples which are the average of nine determinations.

	DSF	DDTC	DDTC-Me	DEA	CS_2
Plasma	51 ± 3.8	85 ± 10.1	91 ± 3.1	52 ± 5.6	48.7 ±10.5
Urine	96.2 ± 11.1	71.2 ± 9.2	92.5 ± 2.5	45.2 ± 5.5	55.4 ± 3.8
Buffer	98.4 ± 4.7	94.9 ± 7.0	96.9 ± 4.4	85.8 ± 5.2	97.4 ± 8.2

TABLE II

REPRODUCIBILITY OF EXTRACTION

Values represent the average of ten determinations ± S.E., except for DDTC which is the average of nine determinations.

Drug entity	Amount added (μg/ml)	Amount found (μg/ml)
DSF	0.5	0.5 ± 0.04
DDTC	0.5	0.5 ± 0.11
DDTC-Me	0.5	0.5 ± 0.07
DEA	0.5	0.48 ± 0.18
CS_2	5.0	4.8 ± 0.38

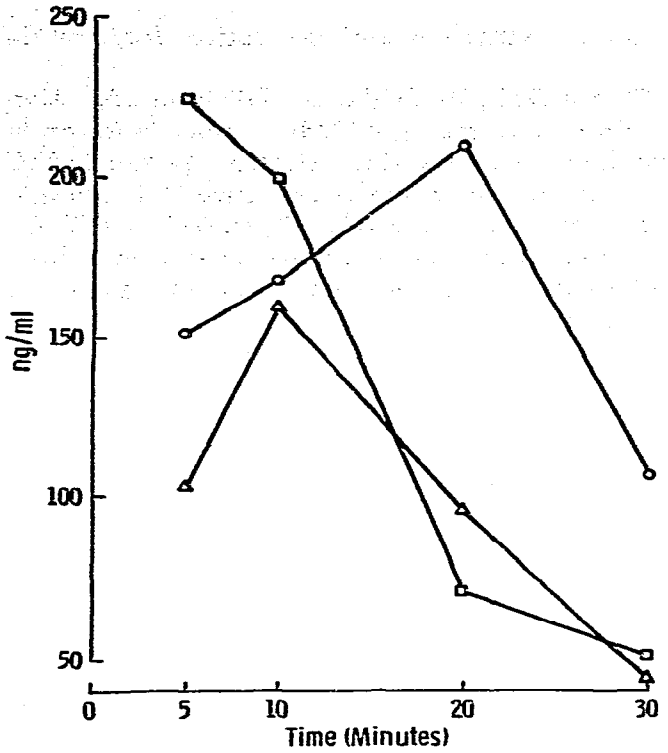

Fig. 4. Plasma concentration of DSF (□), DDTC-Me (○) and DDTC (△) as a function of time after i.p. injection of DSF (200 mg/kg) to mice.

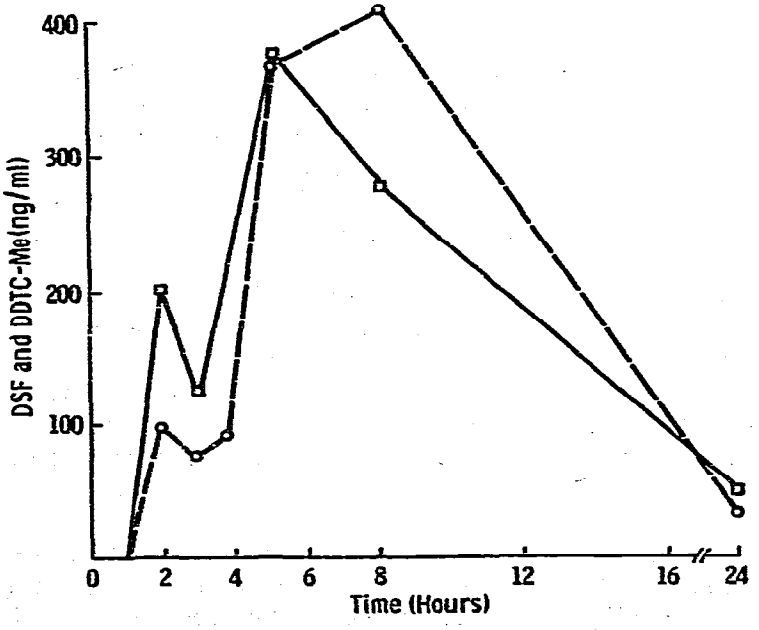

Fig. 5. Plasma DSF (□) and DDTC-Me (○) as a function of time after a single dose of 500 mg of DSF (Antabuse) to an alcoholic volunteer.

ably to that found after subsequent extraction and correction for protein binding.

The plasma concentration profile for DSF, DDTC-Me and DDTC as a function of time after the i.p. administration of 200 mg/kg of DSF to mice is shown in Fig. 4. DSF is detected in plasma 5 min after its administration. Plasma DDTC and DDTC-Me appear to peak 10 and 20 min, respectively, after DSF administration. In Figs. 5 and 6 the plasma concentrations of DSF, DDTC, DDTC-Me, CS_2 and DEA are shown in an alcoholic volunteer at various times after a single dose of orally administered Antabuse. As can be seen, both nanogram and microgram quantities of the drug entities can be found.

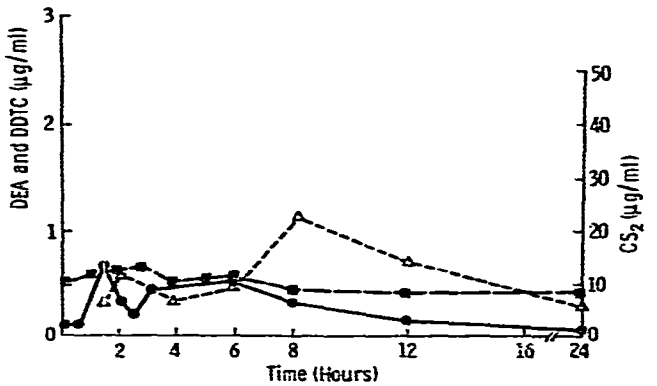

Fig. 6. Plasma concentrations of DEA (●), DDTC (△) and CS_2 (■) as a function of time after a single dose of 500 mg of DSF (Antabuse) to an alcoholic volunteer.

DISCUSSION

The sharpness and symmetry of the peaks shown in Fig. 2 and the linearity of the standard curve in Fig. 3 justify the use of peak height measurements to determine drug concentration. Furthermore, neither plasma nor urine contained endogenous substances which interfered with the HPLC method as the blanks did not show interfering peaks (Fig. 2). In addition, the internal standard employed could be added directly at the extraction step and then extracted along with the other compounds of interest.

The standard curve shown in Fig. 3 was prepared by adding known concentrations of DSF and DDTC-Me to human plasma, and then extracting the drug as previously described. The standard curve gave good linearity in the concentration range 0.2—2.0 µg/ml, with correlation coefficients equal to or greater than 0.995. Intercepts of 15 ng or less were calculated from least-squares regression analysis; this represents an error of 7.5%. The minimum amounts that could be detected in the mobile phase were 2.5 ng for DDTC-Me and 5.0 ng for DSF.

Recovery of DSF and other metabolites added to phosphate buffer was complete (Table I). However, DSF, DEA and CS_2, when added to plasma, and the addition of DEA and CS_2 to urine, showed lower recoveries. It is possible that protein binding may contribute to the lower DSF recovery. This is suggested from preliminary studies where approximately 50% of the DSF

was found bound to bovine serum albumin (unpublished results). This degree of binding to albumin was not found with either DDTC or DDTC-Me. This seems to correlate with the recovery studies for these metabolites as shown in Table I. The lower recoveries of DEA and CS_2 do not appear to be due to volatility, and the reason for this binding at this time is uncertain.

The usefulness of the HPLC method is illustrated in Fig. 4, where the concentration—time profile of DSF, DDTC and DDTC-Me as a function of time after DSF administration to mice is shown. In these experiments, CS_2 and DEA determinations were not made. However, these have been carried out in preliminary studies in alcoholics, and their concentrations can be readily determined. The data illustrated in Fig. 4 show that as the plasma concentration of DSF falls, DDTC increases. The observation that peak plasma levels for DDTC-Me occur after peak levels of DDTC is not unreasonable as methylation of DDTC must first occur. Further discussion concerned with DSF distribution, metabolism and excretion appears elsewhere [22]. In Fig. 5, DSF and DDTC-Me increased gradually after Antabuse, falling to negligible levels after 24 h in this volunteer. Larger quantities of CS_2, DEA and DDTC are observed in plasma (Fig. 6). The plasma levels of DSF found in mice (Fig. 4) and alcoholic volunteers (Fig. 5) appear to be similar, even though the mice received a 30-fold greater dose of DSF. This anomaly is due to the presence of the methylcellulose—polysorbate 80 vehicle which has subsequently been shown to hinder absorption of DSF. In more recent studies with a saline—methylcellulose vehicle in rats this problem has been eliminated.

In developing this HPLC method the effect of pH on extraction was investigated. When the pH was decreased, decomposition of DDTC to CS_2 and DEA occurred. It was found that optimal extraction and minimal decomposition of metabolites occurred at a pH of 8.5.

The accuracy of the analytical method described was tested by adding DSF and its metabolites to plasma (Table II). After extraction, the peak heights of the test samples were compared with those of known standards. The DSF values were corrected for protein binding, while the DEA and CS_2 were corrected for conversion to DDTC-Me which was found to be approximately 95% efficient. The greater variability for CS_2 and DEA may be due to the fact that three extraction steps are required.

The newly developed HPLC method determines DSF and its metabolites with less sample manipulation than most other methods. The method is more sensitive than the colorimetric methods and simpler than those methods requiring the conversion of DDTC to CS_2 and correlation with DSF concentrations. Because DSF can be measured directly, there are fewer problems with the interpretation of data. Furthermore, because of the sensitivity of the analytical method only small samples need be used. This has proved advantageous in clinical studies where multiple samples of 1 ml of plasma have been used.

In conclusion, the HPLC method described fulfils most of the desired criteria. The method is rapid, reproducible, accurate and has the sensitivity needed to determine low concentrations of DSF and its metabolites in biological fluids and tissue. Also, because this is a non-radioactive method, it lends itself to various types of clinical studies.

ACKNOWLEDGEMENTS

This research was supported in part by grants from the National Institute of Neurological and Communicative Disorders and Stroke (NS-07797), the National Institute on Alcohol Abuse and Alcoholism (AA-03577), and by the National Council on Alcoholism, Inc.

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