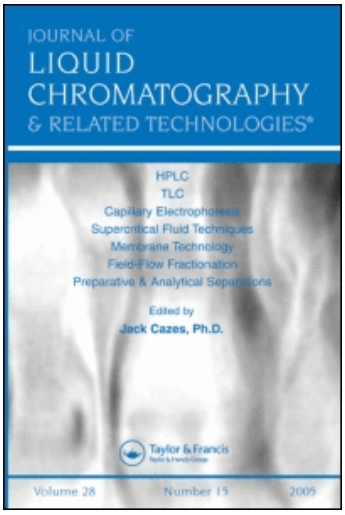

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A Method for the Measurement of total Drug Convertible to Cysteamine: Application to Pharmacokinetic Experiments with Ethiofos

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A METHOD FOR THE MEASUREMENT OF TOTAL DRUG CONVERTIBLE TO CYSTEAMINE: APPLICATION TO PHARMACOKINETIC EXPERIMENTS WITH ETHIOFOS

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ABSTRACT

An analytical method has been developed for the quantification of cysteamine (2-aminoethanethiol) in plasma. A reductive sample pretreatment is used to convert disulfide-bound cysteamine to the free thiol which is subsequently separated by HPLC and detected by electrochemical detection (LCEC). The method was developed to follow drug disposition after administration of ethiofos (WR-2721, *S*-2-(3-aminopropylamino)ethyl phosphorothioic acid) and WR-1065 (2-(3-aminopropylamino)ethanethiol). Standard calibration curves were linear over the range 0.01- to 25.0 μg/mL (0.130-324 μM) and a minimum detectable quantity of 0.01 μg/mL was calculated at a signal-to-noise ratio of 3. Assay precision over the same range averaged 2% (coefficient of variation) and relative recovery, used as a measure of accuracy, was 100%. Stability of cysteamine in plasma, relative to an internal standard (2-aminopropanethiol, WR-186) was good; stored samples were found to contain an average of 94.6 ± 10.4% of the original cysteamine concentration following 28 days at -75 °C.

This method was successfully applied in dosing experiments with rhesus monkeys in which ethiofos was administered into the cephalic vein (IV), portal vein (P) and duodenum. In IV experi-

ments total WR-1065 plasma concentrations greatly exceeded total cysteamine concentrations. While results indicate formation of free and bound cysteamine is not a major metabolic pathway of ethiofos, increased levels of bound cysteamine were observed.

INTRODUCTION

The radioprotective drug ethiofos (*S*-2-(3-aminopropylamino)-ethyl phosphorothioic acid) is known to be rapidly cleaved in the body to its thiol metabolite WR-1065 (2-(3-aminopropylamino)-ethanethiol), the compound thought to be ultimately responsible for the radioprotective effect (1-4). WR-1065 may form a symmetrical disulfide, bis(2-(3-aminopropylamino)ethyl disulfide (WR-33278) or may react with endogenous thiols to form mixed disulfides. We previously reported analytical procedures for the combined measurement of ethiofos, free WR-1065, WR-1065 covalently bound in disulfide form ("total WR-1065") (5) and for free WR-1065 in plasma (6,7).

Studies with [^{14}C]ethiofos indicate further metabolism of WR-1065 (8). Butler et al. (9) reported the appearance of cysteamine mixed disulfides following treatment of fibroblast with WR-1065. The latter group suggest that cysteamine is formed by enzymatic oxidative deamination of WR-1065 to give an aldehyde which then undergoes β-elimination.

Methods have been reported for the determination of free cysteamine. Measurement in plasma was done using amino acid analysis/ion exchange column chromatography (10) but the authors questioned the accuracy of the method. A plasma and urine radioassay for free cysteamine was reported (11) using high-voltage electrophoresis on paper. Free cysteamine in tissues was determined using an enzymatic-spectroscopic technique (12) and HPLC/ECD (13); however, validity of the later method was not established. As part of a program to study the disposition of ethiofos and WR-1065, we developed a method to measure in a single procedure free cysteamine and cysteamine covalently bound in plasma as reducible disulfides. Measurement of "total cysteamine"

may give information useful in the study of ethiofos bioavailability.

MATERIALS

Instrumentation

The HPLC system consisted of an injector (Rheodyne Inc., Cotati, Cal., Model 7125) fitted with a 100-μL loop, pump (Laboratory Data Control, Riviera Beach, Fla., Model Constametric III), a C-18 guard column (30-40 μm Perisorb) and a polyfunctional C-18 chromatographic column (Brownlee Labs, Santa Clara, CA., Model OD-224, 220 mm X 4.6 mm ID, 5 μm Spheri-5). Cysteamine was amperometrically detected at a Hg/Au electrode (Bioanalytical Systems, Inc., West Lafayette, Ind., Model LC-4B) maintained at + 0.15 V vs. Ag/AgCl. A Houston Instruments Omniscribe™ strip chart recorder (Model B5136-5) was used to record the amperometric output from the detector.

Reagents and Solutions

All chemicals were reagent grade or better. The hydrochloride salt of cysteamine (2-aminoethanethiol) was purchased from Aldrich Chemical (Cat. No. 12,292-0). The hydrochloride salt of WR-186 (2-amino-1-propanethiol) (Bottle No. AR 84966) was furnished by Walter Reed Army Institute of Research. Solvents used were HPLC grade and water was treated with a Milli-Q™ purification system (Millipore, Bedford, MA).

Stock solutions (1 mg/mL) containing cysteamine and WR-186 were prepared by dissolving the solid material in 0.1 M chloroacetic acid/0.2 M perchloric acid (1:3) adjusted to pH 3. Fresh stock solutions were prepared weekly and stored at 0-4 °C.

The mobile phase was 0.1 M chloroacetic acid (adjusted to pH 3.0)/acetonitrile (82:18) containing S-12 ion-pair reagent (sodium dodecanesulfonate, Regis Chemical Co., Morton Grove, Ill.) in a concentration of 0.6 mM. Apparent final pH of the mobile phase was 3.4. The eluant was filtered (0.45 μm Nylon-66 filter, Rainin Instrument Co., Inc., Woburn, MA) and degassed under vacuum. Flow rate was maintained at 1.2 mL/min.

METHODS

Sample Preparation

Plasma was obtained from whole blood by centrifugation at 15,600 xG for 1 min. Separated plasma (100 μL) was added to prechilled polyethylene tubes containing either 0.1- or 1.0 μg of the internal standard WR-186 in 200 μL of cold 0.1 M chloroacetic acid/0.2 M perchloric acid (1:3), pH 3.0. The tube contents were mixed for 10 s and rapidly frozen by immersion in a dry ice/2-propanol bath. Samples were stored at -75 °C until time of analysis. While thawing plasma samples at room temperature, the following reagents were added: 200 μL of 30% (v:v) tri-*n*-butylphosphine in chloroform and 200 μL of 0.1 M chloroacetic acid/0.2 M perchloric acid (1:3), pH 3.0. Contents of the sample vial were mixed vigorously for 1 min, placed in a constant temperature bath at 70 °C, and incubated for 30 min. After the sample was cooled in an ice bath and centrifuged for 1 min, 100 μL of the aqueous layer was injected onto the HPLC column.

Four concentrations of cysteamine in plasma were examined in duplicate for relative stability over a 4-week period: 0.01-, 0.1-, 1.0- and 10.0 μg/mL. Samples were prepared by combining 100 μL of the appropriate concentration of cysteamine, 100 μL of a 10.0 μg/mL solution of WR-186 and 100 μL of plasma. Samples were quick-frozen in a dry ice/2-propanol bath and stored at -75 °C until analyzed.

Animal Dosing Experiments (14)

Three healthy male rhesus monkeys (*Macaca mulatta*), 2-3 years old and weighing 3-5 kg, were quarantined 6 weeks for tuberculin testing and were maintained on a recorded regular diet until the time of the experiment. Solid food was withheld from 4 PM before the experiment until 5 PM the day of the experiment and water was given ad libitum. Using ketamine as the anesthetic, a cannula was placed in the femoral vein of each animal approximately 48 hours prior to the dosing study.

Ethiofos (on an anhydrous basis) was administered in each experiment as follows: a 150 mg/kg dose in a 10-mL saline solution into the portal vein over a 10-min period, a 150 mg/kg dose in a 10-mL saline solution into the cephalic vein over a 10-min period and a 300 mg/kg dose in a 5 mL water solution bolus through an infant nasogastric tube placed into the duodenum.

Blood samples (1.2 mL) were withdrawn from the femoral vein and collected in test tubes (Vacutainer™) containing 0.05 mL of a 15% solution of EDTA. Samples were withdrawn at 0-, 0.08-, 0.17-, 0.25-, 0.33-, 0.5-, 0.75-, 1-, 1.5-, 2-, 2.5-, 3-, 4-, 5-, 6-, 8-, 10-, 24-, 32-, 48- and 72 h after drug administration.

RESULTS AND DISCUSSION

Minimum Detectable Quantity (MDQ)

The minimum detectable quantity of cysteamine was initially estimated as 0.01 μg/mL (0.130 μM) based upon analyses over several months. Table 1 lists data from replicate analyses of plasma samples spiked with 0.01 μg/mL cysteamine.

TABLE 1. CALCULATION OF MINIMUM DETECTABLE QUANTITY (MDQ) OF CYSTEAMINE

Peak Height (0.01 μg/mL) Cysteamine (nA)	Average Noise R[a]	MDQ[b] (nA)	MDQ[b] (μg/mL)
0.183	0.0546	0.0425	0.00696
0.196	0.0510	0.0213	0.00326
0.213	0.0469	0.0340	0.00479
0.213	0.0469	0.0340	0.00479
0.209	0.0478	0.0425	0.00609
		$\bar{X}$ = 0.0349	0.00518
		SD = 0.00869	0.00141
	CV (%)	24.9	27.2
	95% Confidence Limit[c]	±0.00998	±0.00162

[a] R = Response Factor = Concentration/Peak Height (nA)

[b] MDQ = S/N × AVG NOISE × R
S/N = 3

[c] 95% Confidence limit = $\pm(t \times SD)/\sqrt{5}$
where t is Student's t (two-sided) with n-1 degrees of freedom

The average MDQ of cysteamine was calculated to be 0.00518 μg/mL using a signal-to-noise ratio (S/N) of 3. Standard deviation was 0.00141 μg/mL and the 95% confidence limit for the MDQ was ±0.00162 μg/mL. Chromatograms of cysteamine (0.01 μg/mL) and blank plasma spiked with WR-186 are shown in Figure 1.

Precision and Accuracy

Values for precision and accuracy were estimated at 0.01-, 0.10-, 1.0-, 5.0-, 10.0-, 20.0- and 25.0 μg/mL cysteamine in plasma and are presented in Table 2. Coefficient of variation (CV) ranged from 1.02 to 3.10% with an average CV of 1.99%. Relative recovery was used as a measure of accuracy and ranged from 99.6 to 100%.

TABLE 2. ACCURACY AND PRECISION OF THE TOTAL CYSTEAMINE METHOD[a]

Prepared Concentration (μg/mL)	Mean Determined Concentration[b] (μg/mL)	Recovery (%)	CV (%)
0.0100	0.0100	100	1.84
0.100	0.100	100	3.10
1.00	1.00	100	1.32
5.00	5.00	100	2.42
10.0	9.96	99.6	1.51
20.0	20.0	100	2.74
25.0	25.0	100	1.02

[a] Determined by response factor

1) $RF = C_{std} \times R_{intstd}/R_{std}$
2) $C_S = RF \times R_S/R_{IS}$

where RF - Response Factor
C_{std} - Cysteamine concentration in calibration standard
R_{intstd} - Response (nA) to internal standard in calibration standard*
R_{std} - Response (nA) to cysteamine in calibration standard
C_S - Concentration of cysteamine in sample
R_S - Response (nA) to cysteamine in sample
R_{IS} - Response (nA) to internal standard in sample

* Concentration of the internal standard was 10 μg/mL in samples and in calibration standards.

[b] Five replicates

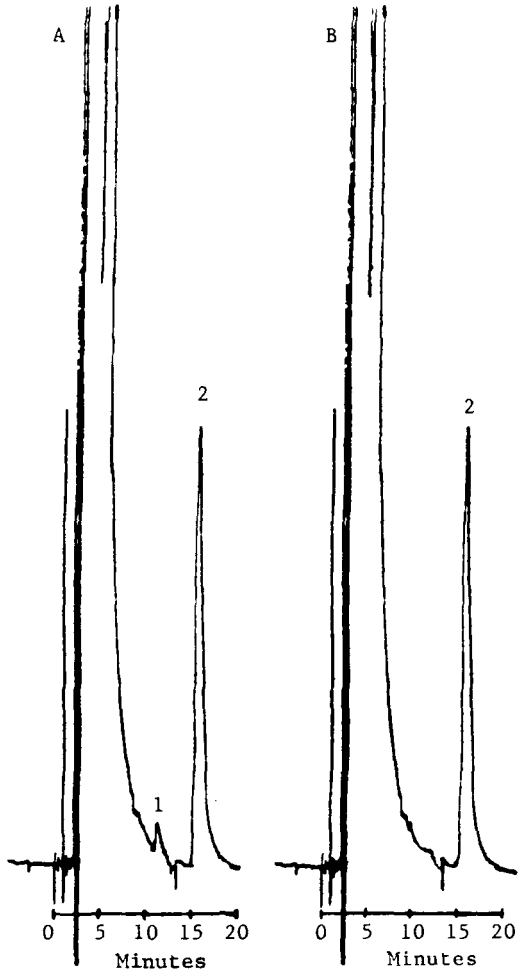

Figure 1. HPLC/ECD of A) plasma calibration standard containing 1) 0.01 µg/mL WR-186 prepared by the total drug convertible to cysteamine method and B) plasma blank from a dosing study by the same method and containing internal standard

Linearity

Plasma standards were prepared to contain 0.01-25.0 μg/mL cysteamine and 10.0 μg/mL WR-186. Standard peak height ratios and total cysteamine concentrations were calculated. Linear fit of plasma concentration data was evaluated using regression analysis of dependent (peak height ratio) and independent (concentration) variables. Detector response to cysteamine was linear over a concentration range of 0.01-25.0 μg/mL (slope: 0.191 mL/μg, intercept: 0.0005, r: 0.9999). Higher concentrations of total cysteamine can be quantitated by diluting the sample to within the linear concentration range. A plasma concentration curve and linear regression are shown in Figure 2.

Stability

Stored and freshly prepared plasma standards were analyzed and peak height ratios of cysteamine and WR-186 were calculated and compared. Stability was expressed as percent of peak height ratio remaining after storage and is given in Table 3.

With the exception of the 0.01 μg/mL 2- and 28-day samples, relative stability of cysteamine was good through 28 days of storage. The average percent of cysteamine (relative to internal standard) remaining after storage in all samples over 28 days was 94.6 ± 10.4%.

TABLE 3. RELATIVE STABILITY OF CYSTEAMINE IN PLASMA EXPRESSED AS PERCENT REMAINING AFTER STORAGE[a,b]

Storage Period (Days)	Concentration (μg/mL) 10	1.0	0.1	0.01
0	93.5 ± 14.1	110 ± 8.48	98.0 ± 0.00	[c]
2	104 ± 5.94	91.5 ± 0.71	86.0 ± 0.00	65.0 ± 7.07
7	107 ± 6.65	101 ± 0.71	105 ± 9.90	95.0 ± 13.5
14	99.6 ± 1.70	101 ± 0.71	103 ± 2.12	100 ± 0.00
21	98.2 ± 1.91	95.0 ± 1.41	83.0 ± 5.66	85.0 ± 7.07
28	92.7 ± 1.98	95.0 ± 1.41	93.0 ± 1.41	75.0 ± 7.07

[a] Each point represents the average of 2 determinations ± SD Cysteamine/WR-186 ratios compared to fresh standard
[b] Samples stored at -75 °C
[c] No determinations made

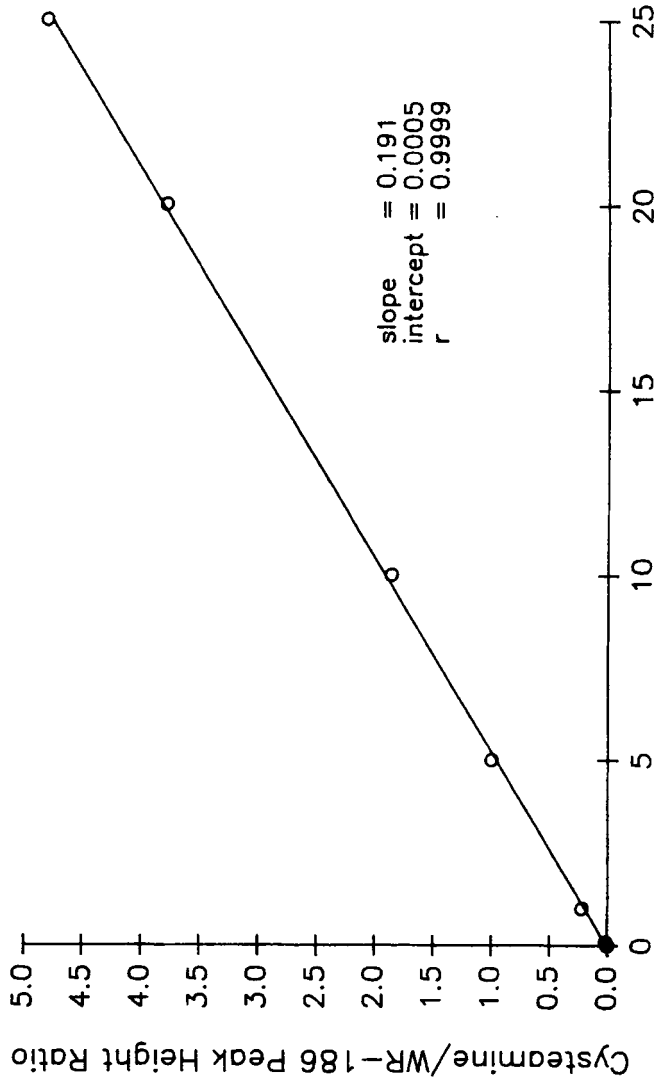

Figure 2. Calibration curve for the total drug convertible to cysteamine method

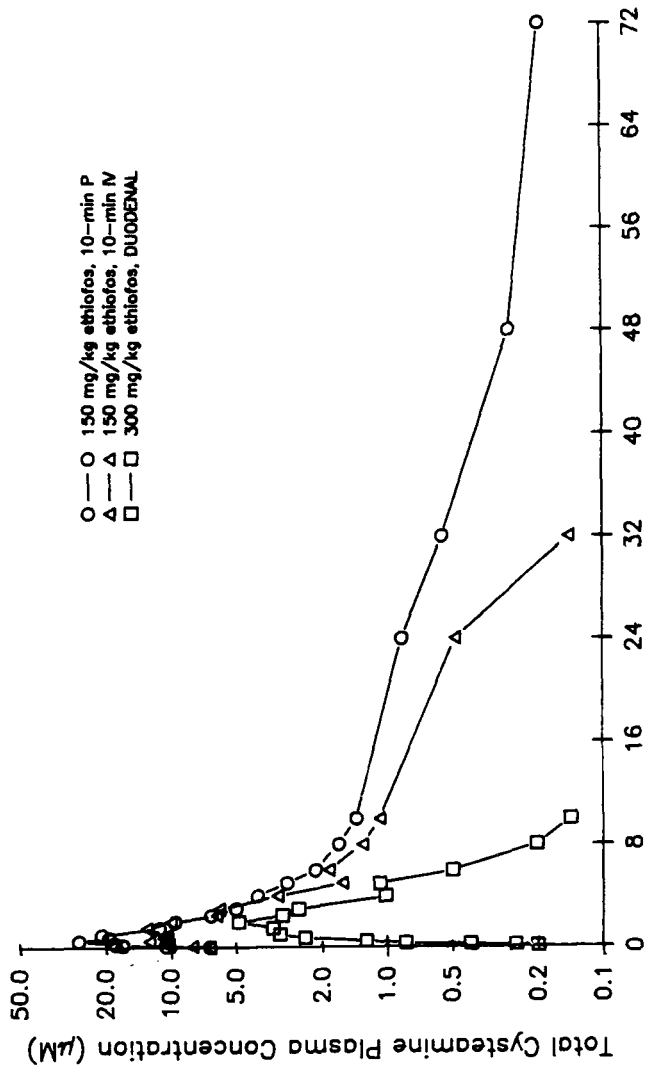

Figure 3. Plasma concentration vs. time profiles for total cysteamine following P, IV and duodenal administration of ethiofos

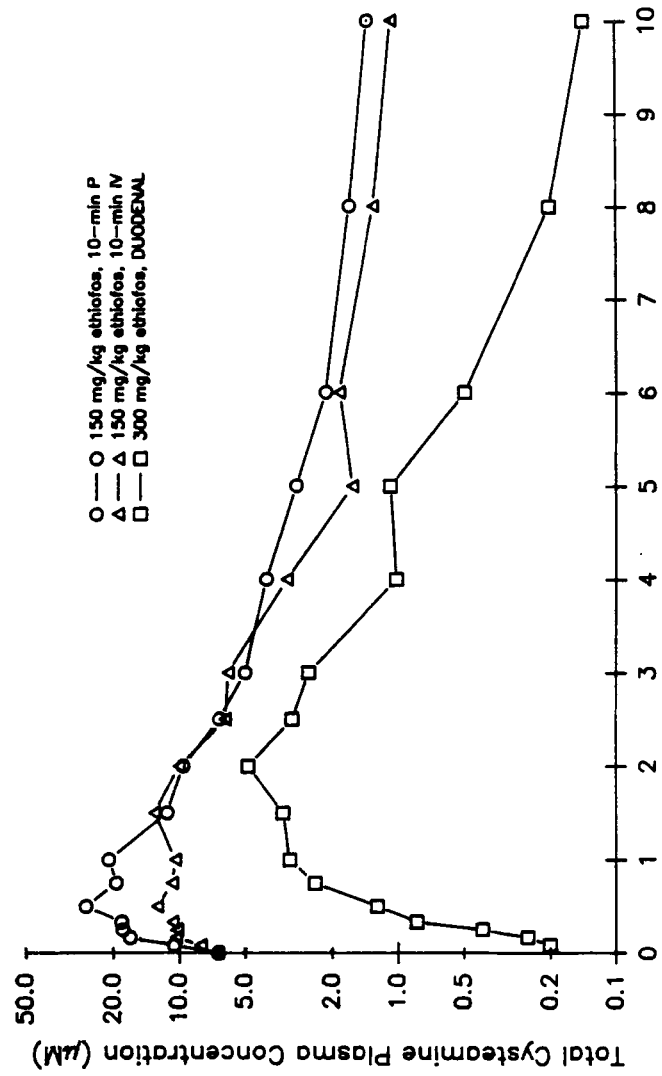

Figure 4. Expanded abscissa of Figure 3

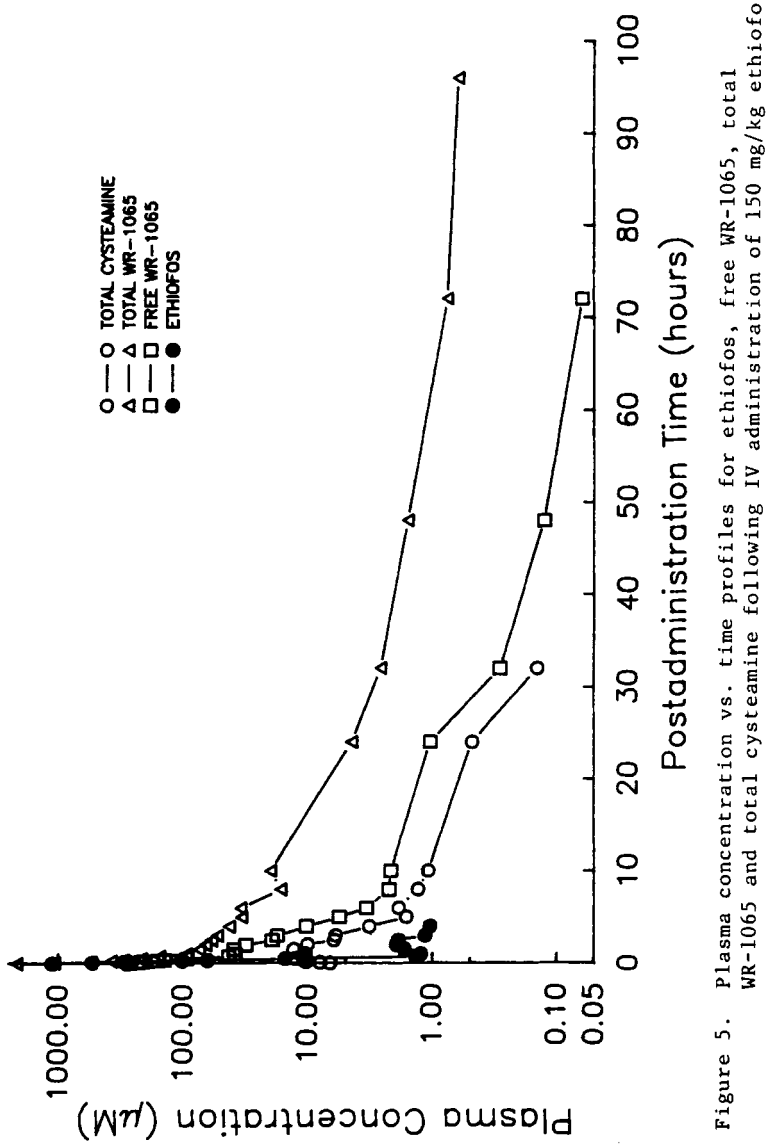

Figure 5. Plasma concentration vs. time profiles for ethiofos, free WR-1065, total WR-1065 and total cysteamine following IV administration of 150 mg/kg ethiofos

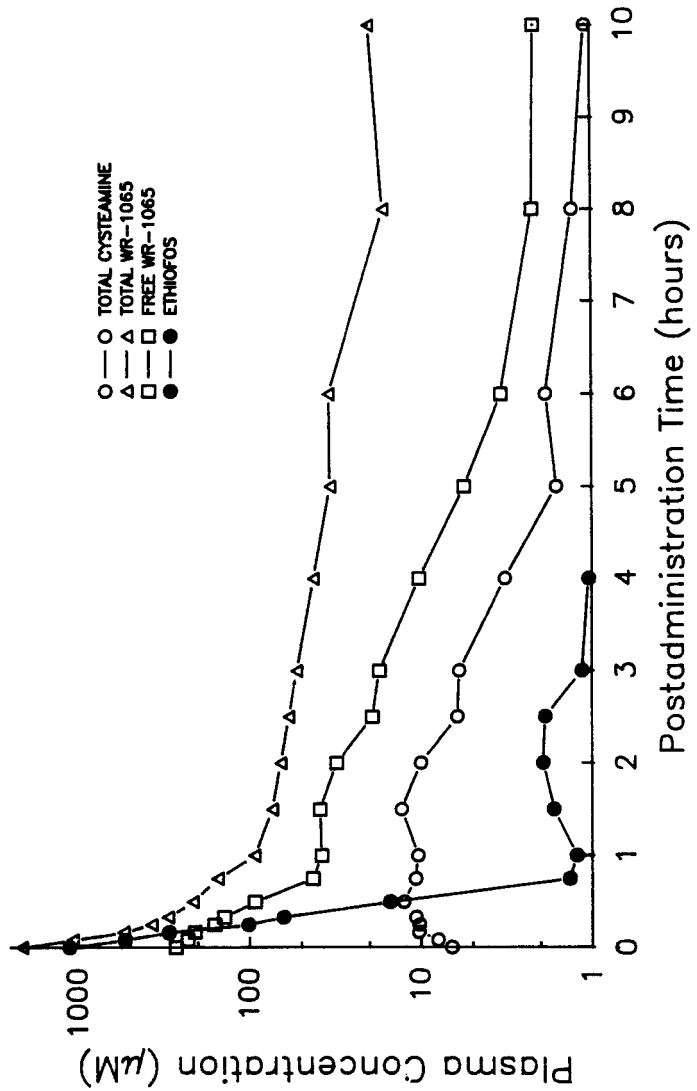

Figure 6. Expanded abscissa of Figure 5

Application

Plasma concentration profiles for total cysteamine following IV, P and duodenal administrations of ethiofos are shown in Figure 3 and 4. P and IV profiles were similar up to 10 h, with concentration maxima of 2.05 and 0.97 μg/mL being reached at 30 min postadministration, respectively. After 10 h total cysteamine concentration declined more rapidly in the IV experiment, reaching the MDQ at 32 h. In the duodenal administration experiment a lower, later maximum (0.37 μg/mL at 2 h) was observed which then steadily declined to the MDQ at 10 h.

Plasma concentration-time profiles for ethiofos, free WR-1065, total WR-1065 and total cysteamine following IV administration of ethiofos are presented in Figure 5 and 6 (expanded abscissa). Plasma concentrations of free and total WR-1065 exceeded total cysteamine concentrations throughout the sampling period. While free WR-1065, total WR-1065 and ethiofos plasma concentrations steadily declined after cessation of infusion, total cysteamine levels continued to rise until 1 h postinfusion then declined. Total cysteamine plasma concentration fell below the detectable limit at 48 h, but total WR-1065 was observed up to 120 h post-administration.

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REFERENCES

1. Purdie, J. W., A comparative study of the radioprotective effects of cysteamine, WR-2721 and WR-1065 in cultured human cells. *Radiat. Res.*, 77, 303, 1979.

2. Purdie, J. W., Dephosphorylation of WR-2721 to WR-1065 in vitro and effect of WR-1065 and misoindazole in combination in irradiated cells, Radiation Sensitizers, Brady, L. W., ed., Masson Publishing, New York, 1980, p. 330.

3. Purdie, J. W., Inhaber, E. R., Schneider, H. and Labelle, J. L., Interaction of cultured mammalian cells with WR-2721 and its thiol, WR-1065: Implications for mechanisms of radioprotection, *Int. J. Radiat. Biol.*, 43, 517, 1983.

4. Mori, T., Watanabe, M., Horiwaka, M., Nikaido, P., Kimura, H., Abyama, T. and Sugahara, T., WR-2721, its derivatives and their radioprotective effects on mammalian cells in culture, *Int. J. Radiat. Biol.*, 44, 41, 1983.

5. Swynnerton, N. F., Huelle, B. K., Mangold, D. J. and Ludden, T. M., A method for the combined measurement of ethiofos and WR-1065 in plasma: Application to pharmacokinetic experiments with ethiofos and its metabolites, *Int. J. Radiat. Oncol. Biol. Phys.*, 12, 1495, 1986.

6. McGovern, E. P., Swynnerton, N. F., Steele, P. D. and Mangold, D. J., HPLC assay for 2-(3-aminopropylamino)ethanethiol (WR-1065) in plasma, *Int. J. Radiat. Oncol. Biol. Phys.*, 10, 1517, 1984.

7. Swynnerton, N. F., Huelle, B. K., McGovern, E. P. and Mangold, D. J., Measurement of WR-1065 in plasma: Preliminary Pharmacokinetics in the beagle, *Res. Comm. Chem. Path. Pharm.*, 54, 255, 1986.

8. Miller, M. A., Swynnerton, N. F., Mangold, D. J. and Ludden, T. M., Disposition of ^{14}C-ethiofos (^{14}C-WR-2721) in the rhesus monkey: Presentation at the 34th annual Radiation Research Society meeting, Las Vegas, Nevada, April 17,1986.

9. Butler, J. D., Gahl, W. A., Tietze, F., Cystine depletion by WR-1065 in cystinotic cells: Mechanism of action, *Biochem. Pharm.*, 34, 2179, 1985.

10. Hsiung, M., Yeo, Y. Y., Itiaba, K. and Crawhall, J. C., Cysteamine, penicillamine, glutathione, and their derivatives analyzed by automated ion exchange column chromatography, *Biochem. Med.*, 19, 305, 1978.

11. Jonas, A. M. and Schneider, J. A., A simple, rapid assay for cysteamine and other thiols, *Anal. Biochem.*, 114, 429, 1981.

12. Ricci, G., Nardini, M., Chiaraluce, R., Dupre, S. and Cavallini, D., Detection and determination of cysteamine at the nanomole level, *J. Appl. Biochem.*, 5 ??0, 1983.

13. Dupuy, D. and Szabo, S., Measurement of tissue sulfhydryls and disulfides in tissue protein and nonprotein fractions by high performance liquid chromatography using electrochemical detection, *J. Liq. Chrom.*, 10, 107, 1987.

14. The laboratory animal facilities and animal care program at Southwest Research Institute has been reviewed and accredited by the American Association for Accreditation of Laboratory Animal Care according to standards set forth by the "Guide for the Care and Use of Laboratory Animals," NIH Publication 85-23.