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GAS CHROMATOGRAPHIC ANALYSIS OF TRIETHYLENETHIOPHOSPHORAMIDE AND TRIETHYLENEPHOSPHORAMIDE IN BIOLOGICAL SPECIMENS

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SUMMARY

Comprehensive pharmacokinetic studies could realise a greater potential for the anti-tumour agent triethylenethiophosphoramide (ThioTEPA), and these would be aided by the development of a selective and sensitive assay. After extraction of ThioTEPA and its metabolite, triethylenephosphoramide (TEPA), from plasma using Sep-Pak C_{18} cartridges, the compounds were separated by capillary chromatography, detected using a nitrogen detector and quantified by reference to an internal standard, hexaethylphosphoramide. The limits of sensitivity were 1—5 ng/ml. Analytical recoveries were 74 and 95%, for TEPA and ThioTEPA, respectively, in the therapeutic range. At similar concentrations, extents of protein binding, determined by ultrafiltration, were not significant. Preliminary investigations of the elimination of ThioTEPA show that drug loss occurs more quickly in mice than in humans and in both species the metabolite is extensively recycled.

INTRODUCTION

The cytotoxic drug triethylenethiophosphoramide (ThioTEPA, Fig. 1), is being used currently in a controlled randomised trial to examine the contri-

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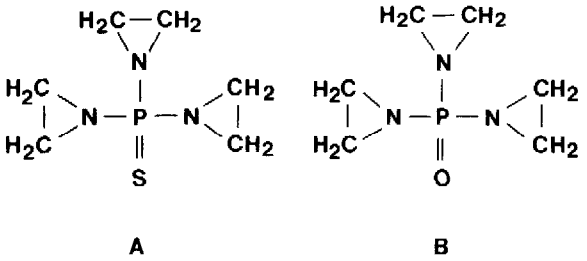

Fig. 1. Molecular structures of (A) ThioTEPA and (B) its metabolite, TEPA.

bution of nandrolone decanoate (Deca-durabolin, Organon) to chemotherapy regimes for ovarian and breast cancer. Studies, both clinical [1] and with animal models [2], indicated that anabolic steroids may stimulate regeneration of peripheral leukocytes. A generalised anabolic effect, however, may not account completely for the increased therapeutic index [1]. It is possible that the synergism of the combination could be explained in part by a pharmacokinetic interaction. For purposes of this investigation a sensitive assay for ThioTEPA and its metabolite, triethylenephosphoramide (TEPA, Fig. 1), also a potent antitumour agent, was required.

Previous studies of the elimination of ThioTEPA were performed using the fluorescent properties of the drug and metabolite [3] or after administration of the isotopically labelled compounds [4—7]. Laborious solvent extractions or chromatographic separations, respectively, were necessary to achieve specificity. Pharmaceutical preparations of ThioTEPA have been analysed using infrared [8] and proton magnetic resonance [9] spectrometry, but these methods are unsuitable for routine determinations of drug levels in plasma. The vapour pressures of TEPA and ThioTEPA, an important consideration in their use as chemosterilants, were determined by gas chromatography (GC) with a flame ionization detector [10].

Retention indices of ThioTEPA have been obtained using various GC conditions and nitrogen detection [11]. Recently, the stability of ThioTEPA in urine and buffer solutions was investigated by solvent extraction of the compound and degradation products before analysis utilizing GC with nitrogen detection [12]. Quantitative determination of TEPA and ThioTEPA in body fluids using GC has not been evaluated previously.

We investigated the application of reversed-phase extraction to sample preparation prior to analysis using capillary chromatography and nitrogen detection. The primary aim was to achieve adequate sensitivity to enable pharmacokinetic characterisation of the drug. Initial application of the method to studies in patients and mice of the elimination of TEPA and ThioTEPA, and their protein binding in vitro is described.

EXPERIMENTAL

Materials

Spectroscopic grade ethanol (BDH, Poole, U.K.) and triple-distilled water were used. Other reagents were of analytical grade. Buffer solutions were phosphate-buffered saline (PBS) which contained $1.34 \cdot 10^{-1}$ *M* NaCl, 5.36 ·

10^{-3} *M* KCl, $1.46 \cdot 10^{-2}$ *M* NaH_2PO_4, $8.16 \cdot 10^{-2}$ *M* Na_2HPO_4, $9.01 \cdot 10^{-4}$ *M* $CaCl_2$ and $1.04 \cdot 10^{-3}$ *M* $MgCl_2$, or 0.5 *M* sodium cacodylate—HCl, pH 7.4.

ThioTEPA was a gift from Lederle Labs. (Gosport, U.K.). TEPA was synthesized by a method similar to that given for the radiolabelled substance by Craig and Jackson [13]; the synthetic compound produced satisfactory analytical and proton magnetic resonance data, and was chromatographically homogeneous. Triethylenemelamine was obtained from Lederle Labs., hexaethylphosphoramide was purchased from Fluorochem (Glossop, U.K.), hexamethylphosphoramide from BDH and tris-(N,N-tetramethylene)-phosphoric acid triamide from Fluka (Buchs, Switzerland); these compounds were examined as possible internal standards for the GC assay.

Human serum albumin (HSA), containing less than 0.005% fatty acid, and human α-, β- and γ-globulins were purchased from Sigma (St. Louis, MO, U.S.A.). Time-expired blood was obtained from the Haematology Department, Bradford Royal Infirmary. Blood was centrifuged at 2000 *g* for 15 min and the separated plasma was stored at −20° C.

Ultrafiltration

TEPA and ThioTEPA (500 mg) were added to PBS (5.0 ml) alone or containing HSA (58.0 mg/ml), α-globulins (15.8 mg/ml), β-globulins (9.3 mg/ml) or γ-globulins (9.6 mg/ml). The mixtures were incubated for 2 h at 37° C and aliquots (1.1 ml) were retained for extraction and GC analysis of total drug or metabolite. The remaining solutions were ultrafiltered at 37° C using a Multimicro concentrator (Amicon, MA, U.S.A.) and Amicon UM10 Diaflo membranes (25 mm diameter). A flow-rate of 0.8 ml/h was maintained using mass flow control of a nitrogen supply. As the initial ultrafiltrate is diluted by buffer solution which remains within the void volume of the membrane after washing, the first portion (500 μl) was discarded and an aliquot (1.1 ml) was then taken for extraction and GC analysis.

Extraction of drug and metabolite

Sep-Pak cartridges containing particles of C_{18}-coated silica (Waters Assoc., Northwich, U.K.) were activated by passing through ethanol (5 ml) and then distilled water (5 ml) under pressure using a plastic syringe. Biological specimens or control samples of plasma (1 ml) were mixed with cacodylate buffer or TEPA and ThioTEPA in cacodylate buffer (100 μl). Hexaethylphosphoramide (50 or 500 ng) in cacodylate buffer or PBS (50 μl) was added to plasma specimens, protein solutions, ultrafiltrates or control samples, which were applied to Sep-Pak cartridges at a flow-rate less than 2 ml/min. The cartridges were washed with distilled water (5 ml) and the solutions remaining were evacuated. Ethanol (2 ml) was passed through the cartridges and the effluents were collected in tapered plastic tubes. Volumes of solvent were reduced to approximately 100 μl by evaporation under a stream of nitrogen with the tubes immersed in a water bath at 55° C. Samples (0.8 μl) were taken up into a gas-tight syringe containing ethanol (0.2 μl) and injected into the chromatograph.

Gas chromatography

GC was performed using a PU4500 capillary chromatograph equipped with

a Grob-type injection system and nitrogen detector (Pye-Unicam, Cambridge, U.K.). Components were separated using a quartz column (25 m × 0.25 mm I.D.), wall-coated (0.2 μm) with SE-30 (Pye-Unicam). Thermal conditions were: injector temperature, 270°C; detector temperature, 300°C; temperature of the column oven was programmed from 180°C to 220°C at 6°C/min. Flow-rates of gases were: flame gases, (hydrogen), 30 ml/min and (air), 300 ml/min; make-up gas (nitrogen), 30 ml/min; carrier gas (hydrogen), 2 ml/min. The split ratio was 5:1.

Patient and animal protocols

Three patients for whom ThioTEPA was part of their medical treatment at the Bradford Royal Infirmary and the Yorkshire Clinic (Bingley, U.K.) participated with informed consent. Each was given the drug (30 mg) as an intramuscular (i.m.) injection. Patients R.A. and L.R. received 150 and 200 mg nandrolone decanoate, two and seven days before ThioTEPA dosage and ampicillin (1 g daily) and dexamethazone (10 mg daily), respectively, were administered concomitantly. Blood specimens (3 ml) were taken by venipuncture into heparinized tubes just before and at 1, 3, 5, 7, 9 and 24 h after dosage.

The experimental animals used were female NMRI mice from our inbred colony. ThioTEPA (10 or 20 mg/kg) was given by intraperitoneal (i.p.) injection. Mice were bled in triplicate by cardiac puncture and the specimens were pooled at hourly intervals during periods up to 12 h after drug administration.

Plasma was separated and stored at −20°C.

Pharmacokinetic analysis

The half-life of elimination ($t_{1/2}$) was determined graphically as the time taken for drug concentration in plasma to decline by 50%. The area under the plasma concentration versus time curve (AUC) was calculated using the trapezoid rule [14].

RESULTS AND DISCUSSION

Analytical considerations

Choice of internal standard. Hexaethylphosphoramide was chosen from the compounds, structurally related to TEPA and ThioTEPA, which were examined as possible internal standards, since it was separated completely from other components in the assay yet eluted within a reasonable time for analysis. Retention times of TEPA and ThioTEPA, relative to hexaethylphosphoramide, were 0.68 and 0.76, respectively. In addition, ThioTEPA had a similar response relative to hexaethylphosphoramide (0.85), but that of TEPA (0.51) was considerably less. Hexamethylphosphoramide fulfilled some of the criteria of a suitable internal standard for TEPA assay, having a similar response and proximate retention time (0.62 and 0.53, relative to hexaethylphosphoramide, respectively), but was rejected on the basis of high volatility [10].

Elution characteristics. The chromatogram shown in Fig. 2A is typical of the fast and efficient separation of components obtained. Retention times of TEPA, ThioTEPA and hexaethylphosphoramide were 3.1, 3.45 and 4.6 min,

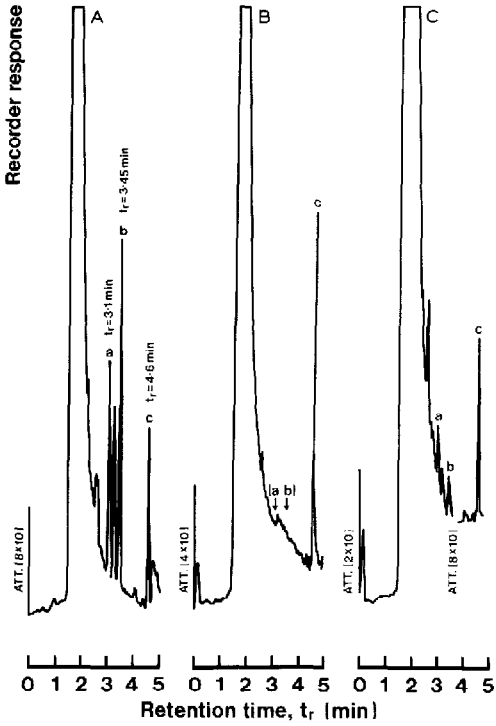

Fig. 2. Gas chromatograms of extracts of plasma (1 ml) containing 50 ng hexaethylphosphoramide: A, control plasma with 100 ng TEPA and ThioTEPA added; B, patient plasma before drug dosage; C, patient plasma, 24 h after i.m. administration of 30 mg ThioTEPA, with concentrations of TEPA (5.3 ng/ml) and ThioTEPA (2.5 ng/ml). Peaks: a = TEPA; b = ThioTEPA; c = hexaethylphosphoramide (internal standard).

respectively. An endogenous component of control human plasma had a retention time intermediate between that of TEPA and ThioTEPA. This extra peak was large in chromatograms of extracts of some specimens of the time-expired plasma used in control experiments (for example, Fig. 2A). The interfering substance, however, was not present in significant quantity in any sample of freshly prepared patient plasma (for example, Fig. 2B) and so did not affect the accuracy of measurement of low levels of drug and metabolite.

Interferences. Nandrolone, the major product in serum after extravascular administration of the decanoate ester, and methotrexate, which is also included often in combination regimes with ThioTEPA, are extracted with greater than 78% efficiency using the Sep-Pak procedure. The cytotoxic drug, its primary metabolite, 7-hydroxymethotrexate, and steroid compounds, however, do not interfere in the GC assay of ThioTEPA, as they are not sufficiently volatile.

Recovery and reproducibility. The mean recovery of hexaethylphosphoramide from plasma using the Sep-Pak procedure and hexamethylphosphoramide as external standard, was 96.4 ± 4.9% (S.E.) at a concentration of 500 ng/ml. Analytical recoveries of TEPA and ThioTEPA and their precision using hexaethylphosphoramide as internal standard are shown in Table I. Recoveries of ThioTEPA were acceptable but those of TEPA were significantly lower, which may reflect in part the more polar nature of the metabolite [3]. The

TABLE I

RECOVERIES OF TEPA AND ThioTEPA FROM PLASMA AND THEIR REPRODUCIBILITIES AFTER SEP-PAK C_{18} EXTRACTION AND GC DETERMINATION USING HEXAETHYLPHOSPHORAMIDE AS INTERNAL STANDARD

Concentration of TEPA or ThioTEPA (ng/ml)	*n*	Recovery (%) (mean ± S.E.)	
		TEPA	ThioTEPA
50.0	12	73.6 ± 5.6*	95.2 ± 7.5*
50.0	12	74.2 ± 6.5**	93.6 ± 7.8**
500.0	6	75.2 ± 5.7*	97.5 ± 5.5*
500.0	6	74.1 ± 7.0**	96.9 ± 8.0**

*Intra-assay statistical variations.
**Inter-assay statistical variations.

greater polarity of TEPA might result in larger binding to active sites on the silica particles of the Sep-Pak material or the metabolite might not partition as strongly to the hydrophobic moiety of the reversed-phase packing, and so may be partly eluted during the aqueous wash. It would be expected, however, that the greater volatility of TEPA than of ThioTEPA [10] would account mainly for the losses of TEPA, which must occur during the concentration stage of sample preparation. Evaporation to dryness of ethanolic solutions of TEPA and ThioTEPA (1 μg/ml), which were then reconstituted in ethanol (100 μl) containing internal standard, resulted in losses of 52.5 ± 4.2% (S.E.) and 34.9 ± 3.9% (S.E.), respectively. According to the method of Dijkhuis [15], isoamylacetate (2.5%, v/v) was added to ethanolic solutions to inhibit evaporation when a volume of 50—100 μl remained. There was no significant increase in recovery of the metabolite or drug and so the ester was omitted and evaporation was performed routinely under supervision until extracts were almost dry.

Linearity. The effect of concentration of TEPA and ThioTEPA on peak height ratio was linear over the ranges, 0—0.2 and 0—1.0 μg/ml, utilizing 50 and 500 ng hexaethylphosphoramide, respectively. Calibration plots, obtained by least squares linear regression analysis of replicate determinations ($n = 6$), were described by: $y = 10.21x - 0.007$ ($r = 0.9999$) for TEPA and $y = 22.38x - 0.03$ ($r = 0.9999$) for ThioTEPA in the lower range of concentration; $y = 1.20x - 0.005$ ($r = 0.9999$) for TEPA and $y = 2.37x - 0.01$ ($r = 0.9999$) for ThioTEPA in the higher range of concentration. Standard deviations in the slopes of the plots were 0.50, 0.88, 0.04 and 0.08, respectively. The total range of the calibration was adequate for estimation of drug and metabolite levels in patient plasma during 24 h after treatment. Because of the greater amounts of the substances in mice, and the lesser volumes of mouse blood obtainable, plasma (100 μl) was diluted to 1 ml with control mouse plasma and estimations were performed over the same range of concentration as for patient samples.

Sensitivity. The lower limit for accurate detection of TEPA and ThioTEPA was 1—5 ng/ml, corresponding to peak height ratios of twice those observed for analyses of control specimens (for example, Fig. 2B). Fig. 2C shows the GC analysis of patient plasma, taken 24 h after ThioTEPA administration, in which concentrations of drug and metabolite are close to the limit of assay sensitivity.

Pharmacokinetic investigations

Humans. Preliminary investigations were performed in three patients after i.m. administration of 30 mg ThioTEPA and these results are shown in Fig. 3. The excretion kinetics of ThioTEPA were apparently first-order in only one patient and the plasma half-life is given in Table II. The elimination kinetics of the metabolite were nonlinear in all cases. Increases in levels of TEPA occurred after its initial peak concentrations were observed and were coincident with the reductions in the rate of ThioTEPA elimination.

Patients who receive nandrolone decanoate are treated using slightly different schedules of combination therapy. In order to assess any possible influence of the steroid on drug or metabolite clearance in a larger study, it will be necessary to determine the plasma levels of nandrolone that exist simultaneously with the measured TEPA and ThioTEPA concentrations. It is intended to implement a radioimmunoassay for this purpose.

Mice. Pharmacokinetic profiles of TEPA and ThioTEPA after administration of higher doses of the drug than were used clinically are shown in Fig. 4. Intraperitoneal administration of 20 mg/kg ThioTEPA resulted in the unusual pattern of metabolite clearance observed in the patient study, but this feature was not so marked at the lower dosage of 10 mg/kg. Significant indications of a dose dependence of ThioTEPA elimination were that on doubling the dose, the half-life did not remain constant but increased two-fold and there was a disproportionate increase in AUC value (Table II).

When administered concomitantly, nandrolone decanoate was shown to

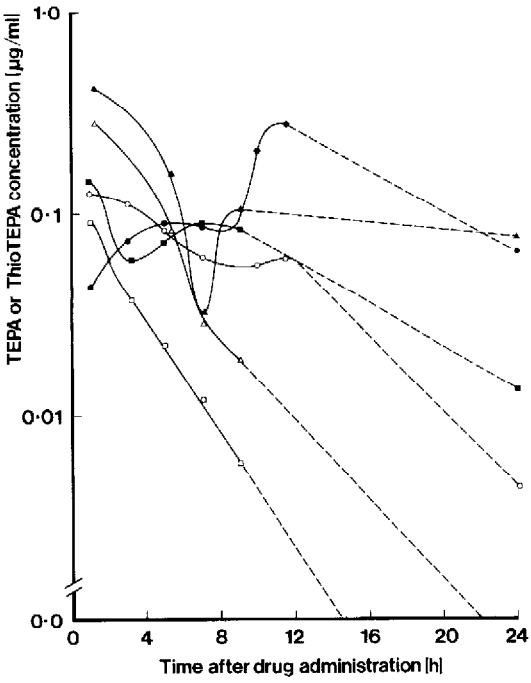

Fig. 3. Plasma levels of drug (open symbols) and metabolite (closed symbols) in patient E.C. (circles), patient R.A. (squares), and patient L.R. (triangles) after i.m. administration of 30 mg ThioTEPA.

TABLE II

ELIMINATION PARAMETERS OF TEPA AND ThioTEPA IN PATIENTS AND MICE

	Dose (mg/kg)	$t_{1/2}$ (min)	AUC* (μg h/ml)		
			TEPA	ThioTEPA	TEPA/ThioTEPA
Patients					
R.A.	0.50	123.0	0.75	0.30	2.54
E.C.	0.32	ND**	0.77	0.76	1.02
L.R.	0.59	ND**	1.76	1.07	1.65
Mice					
	10.0	15.2	25.8***	5.1***	5.06
	20.0	29.1	41.8***	14.7***	2.83
	20.0	28.0	ND§	12.8***	ND§

*Calculated to 9 h after drug dosage.
**Not determined as pharmacokinetics nonlinear.
***Values obtained by interpolating the plasma concentration versus time curve between 0 and 1 h to maxima at 16.0 and 3.3 min for TEPA and ThioTEPA, respectively, as shown in pilot studies of i.p. drug administration in mice.
§Not determined as data inadequate.

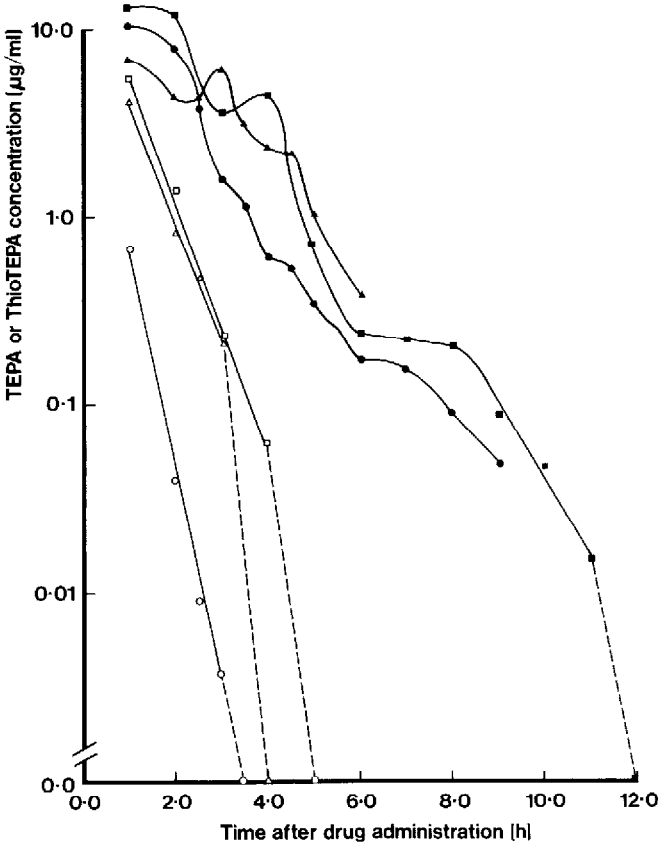

Fig. 4. Plasma levels of drug (open symbols) and metabolite (closed symbols) in three experiments in mice after ThioTEPA administration (i.p.) of 10 mg/kg (circles) and 20 mg/kg (squares and triangles).

significantly decrease the clearance of TEPA but not of ThioTEPA and the reduction was greater in female than in male mice. The detailed results are in preparation for publication.

Species variation. Half-lives of elimination were smaller in mice (Table II) such that drug levels in plasma were undetectable 5 h after dosage. Also, the larger ratios of areas under the drug and metabolite profiles in mice than in patients (Table II) reflect an increased rate of elimination of ThioTEPA rather than decreased clearance of TEPA. In comparison with other species, the mouse is remarkable in its ability to metabolise ThioTEPA completely to inorganic phosphate [7].

Mechanism of elimination. From a comparison of the rate of disappearance of TEPA and ThioTEPA in both patients (Fig. 3) and in mice (Fig. 4), it is apparent that the elimination of TEPA is much slower than that of ThioTEPA. The slower decay of TEPA levels has been observed previously in dogs after an intravenous injection of 3 mg/kg ThioTEPA [3]. As a general rule, lipid solubility is the limiting factor in urinary excretion of drugs [16]. ThioTEPA is more lipid soluble than TEPA, which is extremely soluble in water and would be expected to be excreted more quickly than the parent drug. The fluctuations in the plasma profiles of TEPA particularly in mice (Fig. 4) give some indication of an explanation for reduced metabolite clearance. The declining parts of the curves show irregularly spaced peaks due to loss from and reabsorption into the systemic circulation. The recycling of TEPA may explain the

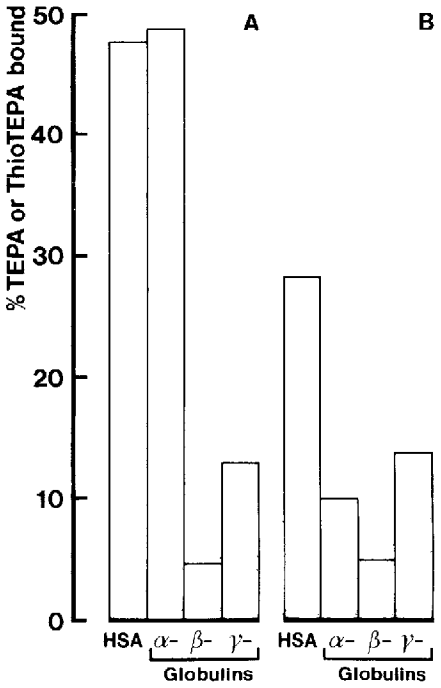

Fig. 5. Extents of binding of A, TEPA and B, ThioTEPA (100 ng/ml) to HSA (58.0 mg/ml), α-globulins (15.8 mg/ml), β-globulins (9.3 mg/ml) and γ-globulins (9.6 mg/ml), corrected for per cent rejection by the membrane.

delayed pharmacological effect of ThioTEPA, which may be manifest in marked myelosuppression up to 30 days after 5—7 day courses [17].

Protein binding. In control experiments in PBS, mean recoveries were 100.5 and 99.2% and intra-assay variations (S.E.) were 6.9 and 2.9% (n = 8) for TEPA and ThioTEPA (100 ng/ml), respectively, after incubation but before ultrafiltration: after ultrafiltration, recoveries were 92.7 and 90.9% and intra-assay variations were 7.7 and 3.9%. These results indicate that TEPA and ThioTEPA solutions at 37°C and pH 7.4 are stable over 2 h and therefore should not degrade to any significant degree during the period of ultrafiltration. In addition, the extents of rejection by the membrane of the drug (8.3%) and metabolite (7.8%) were similar.

The degree of binding of therapeutic concentrations of TEPA and ThioTEPA to human plasma components under physiological conditions are shown in Fig. 5. TEPA was bound to HSA to a greater extent (48%) than ThioTEPA (29%). ThioTEPA did not bind greatly to globulin fractions but the proportion of TEPA bound to α-globulins was significant (49%) by comparison with the degree of binding to β- and γ-globulins (< 15%). Electrophoretic investigations using human plasma in vitro have shown the complete association of ^{14}C-labelled ThioTEPA with protein [4]. Also, greater than 90% of the radioactivity in patient plasma after dosage with ^{32}P-labelled TEPA could be precipitated with 80% (v/v) aqueous acetone [18]. The compounds, therefore, may be associated with protein fractions in addition to those studied here, such as lipoproteins. Contrary to the present findings, Maxwell [19] reported that ThioTEPA was bound extensively and solely to γ-globulins both in vitro and in vivo. Further in vitro studies are required, initially, to clarify the role of the different protein fractions particularly in TEPA binding, before in vivo investigations to ascertain if it has any pharmacological significance.

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REFERENCES

1 R. Turner, Int. Congr. Ser., Excerpta Med. No. 9 (1981) 1.
2 J.A. Double, M.C. Bibby and M.A. Mughal, Brit. J. Cancer, 44 (1981) 305.
3 L.B. Mellett and L.A. Woods, Cancer Res., 20 (1960) 524.
4 J.C. Bateman, H.N. Carlton, R.C. Calvert and G.E. Lindenblad, Int. J. Appl. Radiat. Isot., 7 (1960) 287.
5 L.B. Mellett, P.E. Hodgson and L.A. Woods, J. Lab. Clin. Med., 60 (1962) 818.
6 I.U. Boone, B.S. Rogers and D.L. Williams, Toxicol. Appl. Pharmacol., 4 (1962) 344.
7 A.W. Craig, B.W. Fox and H. Jackson, Biochem. Pharmacol., 3 (1959) 42.
8 U.S. Pharmacopeia XVII, Merck Publishing Co., Easton, PA, 1970, p. 731.
9 L.A. Cates, J. Pharm. Sci., 62 (1973) 1698.
10 D.S. Carlson and D.L. Bailey, J. Agr. Food Chem., 29 (1981) 78.
11 T. Daldrup, F. Susanto and P. Michalke, Z. Anal. Chem., 308 (1981) 413.
12 B.E. Cohen, M.S.B. Nayar, M.J. Elgorin, J. Aisner and P.L. Gutierrez, Proc. Amer. Assoc. Cancer Res., 25 (1984) 287.
13 A.W. Craig and H. Jackson, Brit. J. Pharmacol., 10 (1955) 321.
14 M. Gibaldi and D. Perrier, Pharmacokinetics, Marcel Dekker, New York, 1975, p. 293.

15 I.C. Dijkhuis, in F.W.H.M. Merkus (Editor), The Serum Concentration of Drugs, Clinical Relevance, Theory and Practice, Excerpta Medica, Amsterdam, 1980, p. 53.
16 B.B. Brodie and C.A. Hogben, J. Pharm. Pharmacol., 9 (1957) 345.
17 E.S. Greenwald, NY State J. Med., 72 (1972) 2541.
18 M.V. Nadkarni, E.G. Trams and P.K. Smith, Cancer Res., 19 (1959) 713.
19 J. Maxwell, Ph.D. Thesis, Pharmacology Department, University of Bradford, Bradford, 1969.