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Note

Gas—liquid chromatographic analysis of N,N′,N″-triethylene thiophosphoramide and N,N′,N″-triethylene phosphoramide in biological samples

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N,N′,N″-triethylene thiophosphoramide (Thio-TEPA) is an antineoplastic alkylating agent that has been used clinically for approximately 30 years [1], and which is still utilized extensively in the treatment of metastatic carcinoma of the breast [2, 3], superficial carcinoma of the bladder [4–6] and carcinomatous meningitis [7, 8]. Despite the long history of clinical use of this drug, the plasma pharmacokinetics, tissue distribution and metabolism of Thio-TEPA have remained poorly characterized due to the lack of suitable methodology for analysis of parent compound and its potential metabolites. Earlier studies utilized methods such as radiolabeled drug [9—13], paper chromatography [10, 11], and fluorometric [14] or spectrophotometric assays [15—17] that were cumbersome, relatively insensitive and often non-specific. The recent development by Grochow et al. [18] of a specific and sensitive gas—liquid chromatographic (GLC) analysis of Thio-TEPA, has allowed definition of the concentrations of Thio-TEPA achieved in cerebrospinal fluids of patients receiving intra-thecal Thio-TEPA [18], and of the plasma pharmacokinetics and tissue distribution of Thio-TEPA in mice [19]. Unfortunately, this method is entirely unsuitable for measurement of TEPA, the proposed major metabolite of Thio-TEPA [14, 20], and as such, is not applicable to in vitro and in vivo studies of the biotransformation of Thio-TEPA. This led us to develop a rapid, simple GLC analysis that would overcome the various extraction and chromatographic problems inherent in the existing method and with which we could further explore the metabolism of Thio-TEPA.

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EXPERIMENTAL

Chemicals

Thio-TEPA was graciously provided by Lederle Labs. (Pearl River, NY, U.S.A.) and was proven greater than 99% pure by GLC [19]. TEPA was kindly provided by Dr. George Sosnovsky (University of Wisconsin, Milwaukee, WI, U.S.A.), and was proven greater than 99% pure by the GLC method described below. All other chemicals were of reagent grade unless otherwise specified.

Procedure

Samples (1 ml) of human plasma, containing known concentrations of Thio-TEPA or TEPA, or 1 ml of plasma or urine samples from a patient treated with Thio-TEPA were mixed with 0.1 ml of a 1 mg/ml solution of diphenhydramine (Benadryl, Parke-Davis, Morris Plains, NJ, U.S.A.). Aliquots of 0.1 ml of this mixture were placed into 1.5-ml microcentrifuge tubes which contained approximately 0.2 g of sodium chloride. Chloroform (0.4 ml) was added and the mixture was vortexed and centrifuged for 3 min at 12 000 *g* in a Beckman microcentrifuge 12 (Beckman Instruments, Palo Alto, CA, U.S.A.). The aqueous layer and interface were discarded and 0.3 ml of the chloroform layer was transferred to a fresh tube and dried under nitrogen. The dried residue was reconstituted in 30 μl of ethyl acetate just prior to injection of a 1-μl sample into a Hewlett-Packard 5840A gas chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) fitted with a 1.8 m × 2 mm I.D. glass column containing 3% OV 225 on 100—120 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The oven and injection port were maintained at 180°C and 230°C, respectively. Nitrogen, at a flow-rate of 30 ml/min, was used as the carrier gas. Detection was accomplished with a nitrogen—phosphorous detector that was maintained at 230°C with an air flow-rate of 90 ml/min, a hydrogen flow-rate of 3.5 ml/min, and a bead voltage of 15—18 V. Peaks were traced and integrated with a Hewlett-Packard 5840A GC terminal. Concentrations of Thio-TEPA and TEPA were calculated by comparison with the area of the internal standard peak.

Pharmacokinetic analysis

Computer modeling of the plasma concentrations of Thio-TEPA and TEPA was accomplished with the MLAB program [21] (Division of Computer Research and Technology, NIH, Bethesda, MD, U.S.A.), and pharmacokinetic parameters were calculated from the modeled data.

RESULTS

Diphenhydramine was selected as an internal standard after discovering that diphenylamine, the internal standard used by Grochow et al. [18], co-chromatographed with TEPA. The extraction procedure with chloroform and sodium chloride was selected after systematic demonstration that TEPA was not extracted from plasma by ethyl acetate (the solvent utilized by Grochow et al. [18]), ethyl acetate with sodium chloride, or chloroform without sodium chloride.

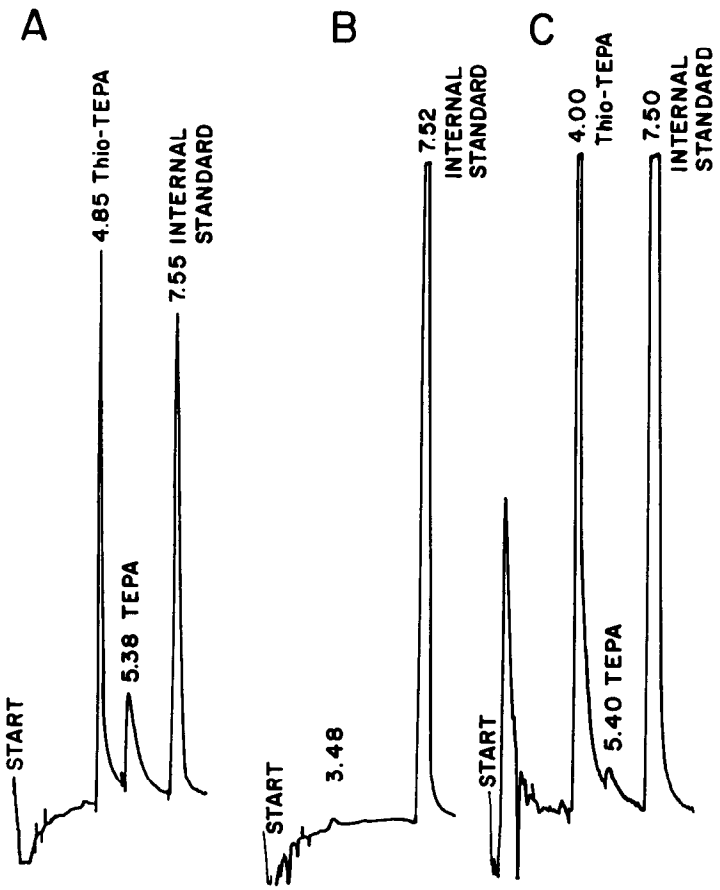

Fig. 1. Chromatograms of control plasma to which was added Thio-TEPA, TEPA and diphenhydramine, internal standard (A) and of plasma from a patient before (B) and after (C) intravenous injection of Thio-TEPA at a dosage of 12 mg/m² of body surface area.

With the extraction and chromatographic conditions described herein, Thio-TEPA and TEPA exhibited two separate peaks which were well resolved from each other as well as from diphenhydramine, the internal standard (Fig. 1). The retention times of Thio-TEPA, TEPA, and diphenhydramine were 4.05, 5.30 and 7.55 min, respectively. There were no endogenous plasma materials which interfered with determination of any of these peaks (Fig. 1) and the limits of detection were 0.01 and 0.1 μg/ml for Thio-TEPA, and TEPA, respectively. When peak area ratios were used to evaluate detector response and to generate standard curves, a linear relationship was obtained over the concentration ranges of 0.01—10 μg/ml and 0.1—100 μg/ml for Thio-TEPA and TEPA, respectively (Fig. 2). For plasma containing known concentrations of drug, the coefficients of variation were 8.6% and 6.0% of the various concentrations, for Thio-TEPA and TEPA, respectively. When standard curves were analyzed by linear regression, correlation coefficients were greater than or equal to 0.98 for both Thio-TEPA and TEPA.

To validate the applicability of this GLC method to biological samples, it was used to analyze plasma and urine samples from a 64-year old white female

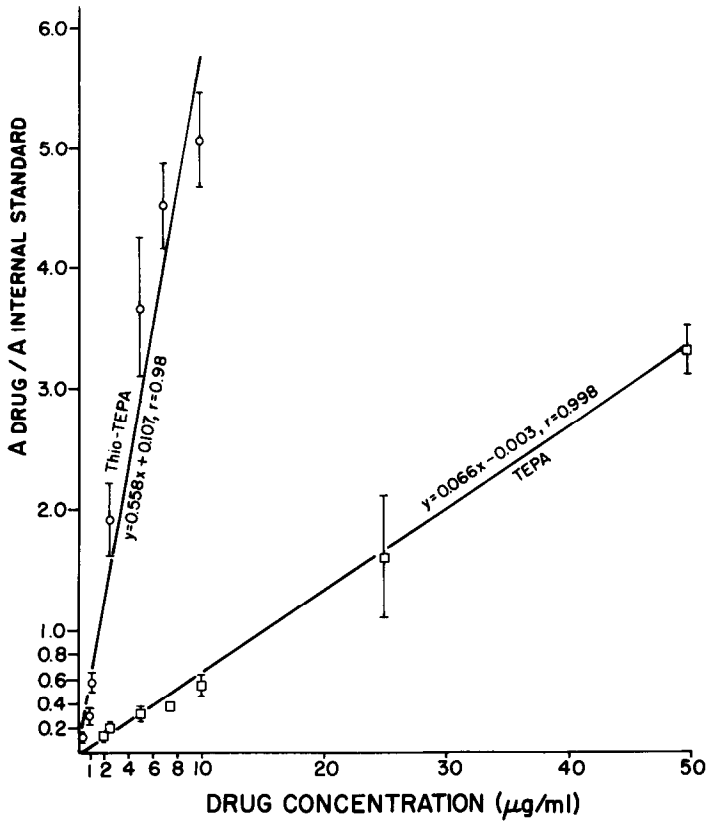

Fig. 2. Standard curve of Thio-TEPA and TEPA. Points represent the means ± standard deviations of TEPA (□—□) and Thio-TEPA (○—○). TEPA: y = 0.0066x — 0.003, r = 0.998; Thio-TEPA: y = 0.558x + 0.107, r = 0.98.

with breast cancer who had received an intravenous (i.v.) bolus injection of Thio-TEPA at a dosage of 12 mg/m^2 of body surface area. Heparinized, venous blood samples were collected prior to Thio-TEPA injection and at 5, 10, 15, 30, 45, 60, 90, 120, 180 and 240 min after injection. Plasma, obtained by centrifuging the blood at 1000 *g* for 10 min, was immediately frozen, and stored overnight at —20°C until analyzed. Urine was collected on ice as voided and was stored frozen at —20°C until analyzed.

Plasma concentrations of Thio-TEPA declined in a biexponential fashion with alpha and beta half-lives of 3.4 and 73.7 min, respectively (Fig. 3). This decline in plasma concentrations of Thio-TEPA was well described by the equation: $C_t = 4.97\ e^{-0.206t} + 0.52\ e^{-0.0094t}$ where C_t represents the concentration at any time t (Fig. 3). Comparison of plasma Thio-TEPA concentrations as defined by this method with those determined on the same plasma samples by the method of Grochow et al. [18], revealed reasonably good agreement (Fig. 3). Further pharmacokinetic analysis of the data generated from this patient indicated an area under the plasma Thio-TEPA concentration vs. time curve (AUC) of 79.6 μg/ml min, and from the relationship, total body clearance = dosage/AUC, a total body clearance of 152 ml/min/m^2 can be calculated for Thio-TEPA. In addition to Thio-TEPA, the patient's plasma con-

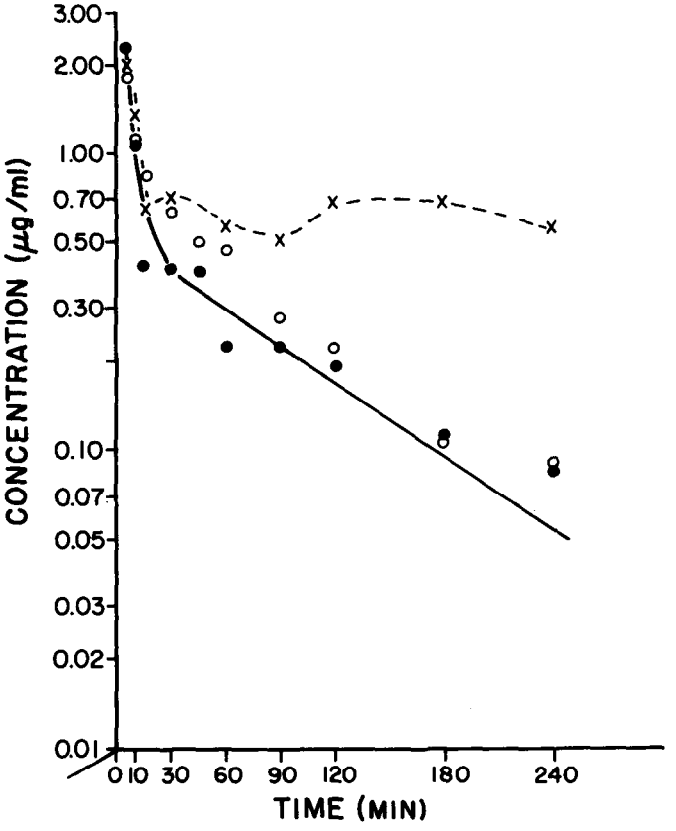

Fig. 3. Concentrations of Thio-TEPA and TEPA in the plasma of a 64-year old female patient with breast cancer who was treated with Thio-TEPA as an i.v. bolus injection at a dosage of 12 mg/m² body surface area. (•) Thio-TEPA concentrations defined by the method described in this manuscript; (○) Thio-TEPA concentrations by the previously described method [18]; (X) Tepa concentrations: the line represents that defined by computer simulation of plasma concentrations of Thio-TEPA (○).

tained substantial concentrations of TEPA (Fig. 3) which, after an initial decline, remained relatively stable from 30 to 240 min after drug administration. Between 0 and 12 h after Thio-TEPA injection, urine contained barely detectable concentrations of Thio-TEPA but concentrations of TEPA were 1—3 μg/ml. There were no endogenous substances in urine which interfered with determination of either substance.

DISCUSSION

The recent publication by Grochow et al. [18] of a rapid, sensitive, and selective GLC assay for Thio-TEPA has allowed new investigations by our laboratory and others into the pharmacokinetics and tissue distribution of this agent [18, 19, 22]. However, our efforts to investigate, in vitro and in vivo, the metabolism of this widely utilized antineoplastic agent were hampered by the inability to detect and quantify TEPA, which is not only the proposed major metabolite of Thio-TEPA in man [14, 21, 23] but is itself an active, anti-

proliferative compound [22, 24–26]. Systematic evaluation of this problem revealed defects both in the nature of the internal standard and in the extraction procedure employed previously [18, 19]. The method presented in this manuscript, although slightly more involved than that previously described, is still rapid, sensitive and reproducible. Moreover, the ability to detect and quantify both TEPA and Thio-TEPA is essential for documentation of the conversion of Thio-TEPA to TEPA and for the investigation of the enzymatic pathways involved in this biotransformation. The plasma pharmacokinetic data from our patient, as presented in this manuscript, not only confirms the applicability to biological samples of the method described but clearly documents the presence of substantial concentrations of TEPA in the patient's plasma and urine. Studies currently underway in our laboratory are extending these observations by utilizing the method described in this communication to characterize more fully the human plasma pharmacokinetics of Thio-TEPA and to characterize the enzymatic mechanisms responsible for metabolism of Thio-TEPA [27] to TEPA.

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