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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE ANTI-TUMOUR AGENT TRIETHYLENETHIOPHOSPHORAMIDE AND ITS METABOLITE TRIETHYLENEPHOSPHORAMIDE WITH SODIUM SULPHIDE, TAURINE AND o-PHTHALALDEHYDE AS PRE-COLUMN FLUORESCENT DERIVATIZATION REAGENTS

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SUMMARY

The method described is based on the reaction of triethylenethiophosphoramide (ThioTEPA) and triethylenephosphoramide (TEPA), through their ethyleneimine groups, with sodium sulphide, taurine and o-phthalaldehyde to give fluorescent products, and separation of the derivatives by reversed-phase high-performance liquid chromatography. The method was successfully applied to the determination of ThioTEPA and TEPA in rabbit plasma samples after clean-up with an Extrelut 3 column. The recoveries of ThioTEPA and TEPA from plasma were 66.1–80.3% and the limits of determination in plasma were *ca.* 10 and 20 ng/ml, respectively.

INTRODUCTION

The alkylating antitumour agent triethylenethiophosphoramide (ThioTEPA) (Fig. 1) has been used in cancer treatment. ThioTEPA is metabolized to triethylenephosphoramide (TEPA) (Fig. 1), which is also an alkylating agent. Because of their high cytotoxicity, the simultaneous determination of these two compounds is desirable for pharmacokinetic studies[1]. Previously reported methods, such as spectrophotometric[2] and fluorimetric[3] methods, did not distinguish between the parent drug and its metabolite unless a cumbersome procedure[3] was used. An assay method based on the use of radiolabelled drugs[4] is not easy to perform. Recently, gas
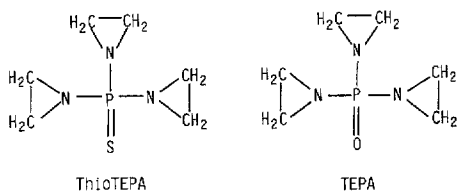

Fig. 1. Structures of ThioTEPA and TEPA.

chromatography (GC) with nitrogen–phosphorus detection[5,6] was reported. The method is very sensitive and permits the determination of both ThioTEPA and TEPA. The GC method, however, requires a high operating temperature and accordingly it may cause undesirable problems in biological analysis because of the thermal instability of ThioTEPA and TEPA and many endogenous substances which can inactivate the column packings.

Despite the utility of high-performance liquid chromatography (HPLC) for determining thermally unstable compounds, no application of HPLC for ThioTEPA and TEPA has been reported so far, perhaps because they have no specific ultraviolet-active or fluorescent functional groups in their molecule. Recently, a fluorigenic reaction of ThioTEPA, based on the S-alkylation reaction of its ethyleneimine group with sodium sulphide and subsequent condensation with o-phthalaldehyde (OPA) and taurine was reported[7]. This reaction seemed promising as a method for the pre-column derivatization of ThioTEPA and TEPA for HPLC. This work was aimed at devising an HPLC method for the simultaneous determination of ThioTEPA and TEPA by using the fluorigenic reaction described above. An application to rabbit plasma was also examined.

EXPERIMENTAL

Chemicals and materials

All chemicals were of analytical-reagent grade, unless stated otherwise. Water was purified on a Milli RO-Milli Q system (Millipore, Bedford, MA, U.S.A.). ThioTEPA was kindly supplied by Simitomo Pharmaceuticals (Osaka, Japan). TEPA was synthesized according to the published method[8] with a slight modification: the crude product obtained was purified on a LiChroprep RP-18 (40–63 μm) column 310 mm × 25 mm I.D. (Merck, Darmstad, F.R.G.) with 30% aqueous methanol as the eluent (flow-rate, 1.0 ml/min). Mass spectra of TEPA gave a molecular weight of 173 (M^+) (calculated for $C_6H_{12}N_3PO$, 173.0717; found, 173.0719). Standard solutions of ThioTEPA and TEPA were prepared in 1-propanol. An Extrelut 3 column was obtained from Merck. The solutions used for the fluorescent derivatization reaction were prepared as follows.

Sodium sulphide (40 mM)–ethylenediaminetetraacetic acid, tetrasodium salt (Na₄EDTA) (50 mM) solution. Equal volumes of aqueous 80 mM sodium sulphide nonahydrate and aqueous 100 mM Na_4EDTA were mixed (prepared daily).

Taurine solution (0.2 mM). Taurine was dissolved to give a 0.2 mM solution in 0.1 M phosphate buffer (pH 8.0).

OPA solution (0.3 mM). OPA was dissolved to give a 0.3 mM solution in 0.1 M phosphate buffer (pH 8.0).

Fluorescent derivatization

To 100 μl of sample solution in a 1.5-ml glass-stoppered test-tube were added 10 μl of sodium sulphide–EDTA solution. The tube was heated in a water bath at 80°C for 30 min and then cooled in an ice–water bath. To the mixture, 400 μl each of taurine and OPA solutions were added about 10 min before injection of the sample (20 μl) into the HPLC system.

HPLC apparatus and conditions

The HPLC system consisted of a Shimadzu LC-6A pump (Shimadzu, Kyoto, Japan), a Rheodyne Model 7125 injector with a 20-μl sample loop (Rheodyne, Cotati, CA, U.S.A.) and a 5-μm Hibar LiChrosorb RP-18 (250 mm × 4 mm I.D.) (Merck) with a guard column (LiChroCART RP-18) (Merck). The mobile phase was 0.1 M phosphate buffer (pH 5.7)–acetonitrile (72:28). The column temperature was ambient and the flow-rate was 1.0 ml/min. Detection was carried out with a Hitachi F-1000 fluorescence spectrophotometer equipped with a flow cell (12 μl) and a xenon lamp (Hitachi, Tokyo, Japan), operated at 440 nm emission and 340 nm excitation.

Extraction of ThioTEPA and TEPA from plasma samples

A plasma sample (1 ml) was diluted with water (2.2 ml) and 3 ml of the solution were applied to the Extrelut 3 column. After 15 min, chloroform was passed through the column. The first 8 ml of effluent were collected and dried under nitrogen at *ca.* 20°C. The residue obtained was dissolved in 500 μl of 1-propanol. A 100-μl aliquot of the solution was then examined by the above methods.

RESULTS AND DISCUSSION

Derivatization

The method used in this study was essentially the same as that described in a previous paper dealing with a manual procedure for the determination of ThioTEPA[7]. TEPA was also derivatized by this method, although its fluorescence intensity was *ca.* 45% of the value for ThioTEPA. The OPA reaction for the products resulting from ThioTEPA and TEPA with sodium sulphide was carried out in the presence of taurine at basic pH. In the manual procedure, borate–phosphate buffer (pH 8.0) was used for the preparation of the OPA and taurine reagents. However, in this study, 0.1 M phosphate buffer (pH 8.0) was used instead of borate–phosphate buffer, because phosphate buffer was used as a component of the eluent for HPLC. The fluorophores obtained by the proposed method are assumed to be isoindole derivatives, which are known to be very unstable[9,10]. The final fluorescence obtained for ThioTEPA and TEPA was stable for 20 min at room temperature. On the other hand, alkylated products of ThioTEPA and TEPA with sodium sulphide were stable for at least 8 h in an ice water bath. Therefore, after the alkylation reaction, the reaction mixture was stored in an ice–water bath without addition of taurine and OPA reagents. Both of the reagents were added about 10 min prior to injection of the sample into the HPLC system, because a reaction time of 10 min at room temperature was required to give the maximum fluorescence intensity.

Separation

The simultaneous separation of the fluorescent derivatives was studied to revesred-phase HPLC, because the derivatization reaction was carried out in aqueous solution. Also, isoindole fluorophores are known to separate efficiently on reversed-phase columns with a mobile phase consisting of aqueous methanol[11] or acetonitrile[12] containing phosphate or acetate buffers. Therefore, phosphate buffer solutions (0.02–0.2 M, pH 5.7–8.0) mixed with acetonitrile (25–30%, v/v) or methanol (50–60%, v/v) were tested as eluents. Although the derivatives of ThioTEPA and TEPA were
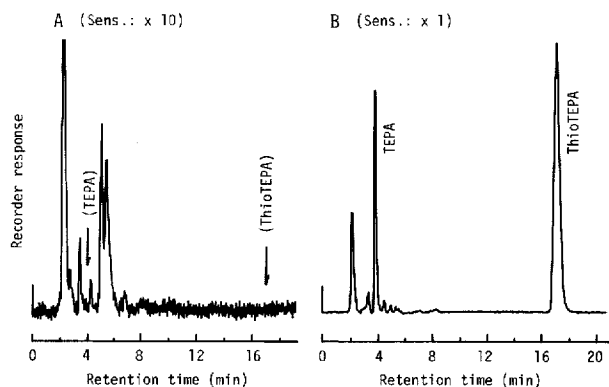
Fig. 2. HPLC traces of (A) reagent blank and (B) derivatives resulting from a standard solution containing ThioTEPA (189 ng) and TEPA (173 ng) in 100 μl of 1-propanol.

easily separated in both instances, significant problems were encountered with the interference of a few minor peaks originating from the reagents. The retention times of the derivatives and reagent blank decreased with either increasing concentrations of organic solvents and pH or decreasing phosphate concentration in the mobile phase. It was found that acetonitrile–0.1 M phosphate buffer (pH 5.7) (28:72) permitted the satisfactory resolution of peaks for TEPA and the reagent blank (Fig. 2). The retention times of ThioTEPA and TEPA were 17.0 and 4.0 min, respectively. Other ionic media, such as borate, acetate and citrate buffers, were examined, but no improved separation from the interfering peaks was obtained.

Calibration graphs for standard solutions

Linear calibration graphs were obtained for ThioTEPA and TEPA over the concentration ranges 1.5–378 ng (8–2000 pmol) and 3.5–346 ng (200–2000 pmol), respectively, in 100 μl of 1-propanol, with a relative standard deviation of *ca.* 5%. When the calibration graphs were analysed by least-squares linear regression, the equations of the lines were $y = 2.84x + 0.39$ ($r = 0.9993$) for ThioTEPA and $y = 2.47x - 0.83$ ($r = 0.9998$) for TEPA. The detection limits were 1.5 and 3.5 ng of ThioTEPA and TEPA, respectively, in 100 μl of 1-propanol at a signal-to-noise ratio of 2.

Clean-up of ThioTEPA and TEPA in plasma samples

In previous work[7] the utility of an Extrelut 3 column for the clean-up of plasma ThioTEPA was demonstrated. It was more effective and easier to use than other methods, such as chloroform extraction[6] and the use of Sep-Pak C_{18} cartridges[5]. The column was therefore examined for the simultaneous extraction of ThioTEPA and TEPA from plasma samples. When rabbit plasma (1 ml) to which 945 ng of ThioTEPA and 865 ng of TEPA had been added was diluted with water (2.2 ml) and then applied to the column, maximum recoveries (*ca.* 70–80%) were obtained by elution with 7 ml of chloroform. Dichloromethane and ethyl acetate gave low recoveries, especially for TEPA (below 50%), and therefore chloroform (8 ml) was used. The use of a stream of nitrogen is recommended instead of a rotary evaporator for evaporating the eluent.
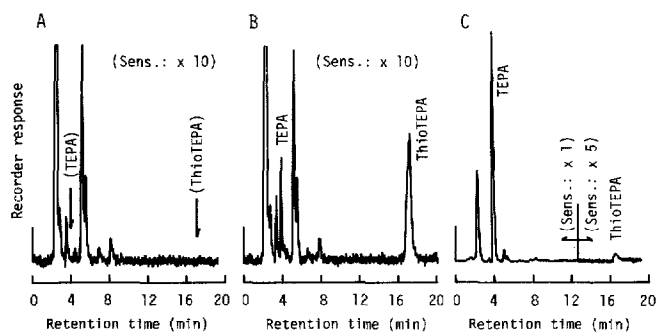
Fig. 3. HPLC traces of extracts of rabbit plasma: (A) control plasma; (B) control plasma with ThioTEPA (94.5 ng) and TEPA (86.5 ng) added; (C) plasma sample obtained 1 h after intravenous injection of 1 mg/kg of ThioTEPA, containing 8 ng/ml of ThioTEPA and 1250 ng/ml of TEPA.

The latter gave poor reproducibility of the recovery of TEPA, probably because of the greater volatility of TEPA. As shown in Fig. 3, this clean-up procedure was effective for the removal of endogenous substances, which could interfere with the derivatization reaction and the chromatographic separation. For example, the HPLC trace resulting from blank plasma (Fig. 3A) was essentially the same as that of the reagent blank itself (Fig. 2A). ThioTEPA and TEPA were detected successfully without interferences (Fig. 3B and C). From the chromatogram of plasma to which were added ThioTEPA (94.5 ng) and TEPA (86.5 ng), the limits of determination were estimated to be ca. 10 and 20 ng/ml for ThioTEPA and TEPA, respectively, in plasma (Fig. 3B). Although significant losses of ThioTEPA and TEPA occurred during the evaporation stage, owing to their volatility, about 66–80% recoveries were obtained for ThioTEPA and TEPA added to plasma at concentrations of 86.5–945 ng/ml (Table I). The standard deviations were also satisfactory. By employing a clean-up procedure similar to that in the present method, we previously obtained a linear calibration graph for plasma ThioTEPA[7]. Therefore, this method presumably permits the determination of ThioTEPA and TEPA at concentrations up to ca. 2500 ng/ml in plasma with a mean recovery of ca. 70% (corresponding to 330 ng per 100 μl in the final solution after dissolving the dried residue in 500 μl of 1-propanol).

TABLE I

RECOVERIES OF ThioTEPA AND TEPA FROM RABBIT PLASMA

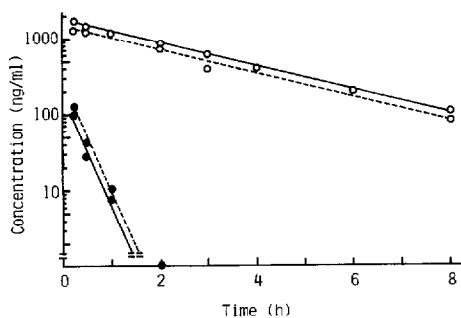| Experiment No.	ThioTEPA or TEPA added [nmol/ml (ng/ml)]	Recovery (%)*
1	ThioTEPA 0.5 (94.5)	66.1 ± 8.2
	TEPA 0.5 (86.5)	72.3 ± 4.6
2	ThioTEPA 5 (945)	80.3 ± 9.8
	TEPA 5 (865)	72.1 ± 8.4

* Mean ± standard deviation ($n = 4$).

Fig. 4. Plasma concentrations of (●) ThioTEPA and (○) TEPA in two rabbits (solid line, 3.7 kg; broken line, 3.8 kg) following a single intravenous injection of 1 mg/kg of ThioTEPA.

Determination of ThioTEPA and TEPA in rabbit plasma

In order to test the applicability of the proposed method to plasma samples, it was used to analyse plasma from two male rabbits (3.7 and 3.8 kg) that had been given an intravenous injection of a ThioTEPA preparation (1 mg/kg). Venous blood specimens were collected in heparinized tubes just before and up to 8 h after the drug administration. A typical chromatogram and time courses of plasma levels of the drug and its metabolite are shown in Figs. 3C and 4, respectively. It should be noted that the concentrations of ThioTEPA and TEPA indicated in Figs. 3C and 4 were not corrected for recovery. Plasma concentrations of ThioTEPA and TEPA declined exponentially. By 2 h after injection, there was little ThioTEPA in the plasma, but *ca.* 100 ng/ml of TEPA was detected, even 8 h after injection. These results are similar to those reported for mice[5]. In previous work, 200–400 ng/ml of ThioTEPA were detected, even 5 h after intravenous administration of ThioTEPA to three female rabbits (5 mg/kg). However, as suggested in that paper[7], it seems that the values determined as ThioTEPA must include considerable amounts of TEPA, because it is also eluted from the Extrelut column under the conditions used in the study.

Chromatographic procedures are considered to be the most suitable for the simultaneous determination of ThioTEPA and TEPA in biological fluids. A few methods based on GC with nitrogen–phosphorus detection have been reported[5,6], but apparently no application of HPLC methods has been described. This study provides the first HPLC method for determining ThioTEPA and TEPA. The proposed method permits the determination of 10 and 20 ng/ml of ThioTEPA and TEPA, respectively, in rabbit plasma. This sensitivity is greater than that of the method based on capillary GC with nitrogen–phosphorus detection and temperature-programmed elution (the detection limits were 1–5 ng/ml in human plasma for both compounds)[5], but it is comparable to that obtained by conventional and isothermal GC with nitrogen-phosphorus detection (the detection limits were 10 and 100 ng/ml of ThioTEPA and TEPA, respectively, in human plasma)[6]. Probably there is not a marked difference between the recoveries obtained by our method and by the GC method[5,6], except for ThioTEPA treated by the method of McDermott *et al.*[5] (in which more than 90% recoveries were reported), because in both the GC method and our method the evaporation of the extracts from plasma is necessary in order to improve the sensitivity, and this step causes significant losses of the analytes.

Pharmacokinetic studies of ThioTEPA and TEPA were recently performed with such GC procedures. Probably the HPLC method described here is also capable of determining ThioTEPA and TEPA in plasma samples from patients following a therapeutic dose of ThioTEPA. The method should be useful for clinical studies.

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