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APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH SPECTROPHOTOMETRIC AND ELECTROCHEMICAL DETECTION TO THE ANALYSIS OF ALKYLENEBIS(DITHIOCARBAMATES) AND THEIR METABOLITES

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SUMMARY

The application of high-performance liquid chromatography with spectrophotometric or electrochemical detection was demonstrated for the determination of some alkylenebis(dithiocarbamates) (ABDTCs) and some of their degradation products and metabolites, especially alkylenethiourea, for toxicological investigation. First the ABDTCs were dissolved in the presence of EDTA and antioxidant, followed by ion-exchange chromatography with electrochemical detection (0.1–20 ng injected) or UV detection (20–5000 ng injected). Then normal-phase chromatography with UV detection was performed to determine ethylene- and propylenethioureas (0.1–1000 ng injected). The above-mentioned compounds were determined in technical products, air, blood, tissues, etc.

INTRODUCTION

Alkylenebis(dithiocarbamates) (ABDTCs), being metal (M^+ or M^{2+}) or ammonium salts of propylene- or ethylenebis(dithiocarbamic) acids (**1**–**7**) exhibit fungicidal activity. The M^+ salts are water-soluble, having a high toxicity and short-lived fungicidal action and are not widely used. In contrast, the ABDTCs of some bivalent metal ions, being polymeric products, are practically insoluble, have a low toxicity (LD_{50} = 4000–9000 mg/kg) and durable fungicidal action and are widely used in agricultural practice. However, they are unstable compounds, undergoing degradation to alkylenethioureas[1], the latter being some of the metabolites as well[2]. It has been shown that compounds of this type exhibit a high degree of carcinogenicity, mutagenicity and teratogenicity[2–4]. For that reason, the application of this type of fungicides in practice requires convenient methods for their determination as well as that of their degradation (metabolite) products. However, their analysis is hampered by their low solubility, low stability and polymeric structure. Compounds

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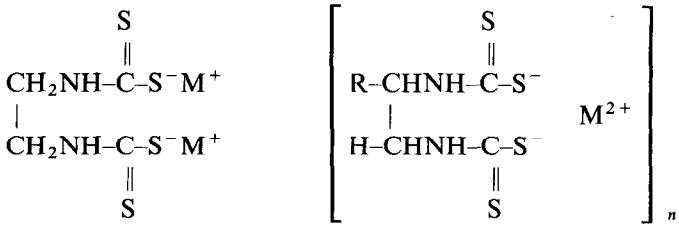


1 $M^+ = Na^+$ (Nabam)
2 $M^+ = NH_4^+$ (Amobam)

3 R = H; M = Zn (Zineb)
4 R = H; M = Mn and Zn (Mankozeb)
5 R = H; M = Mn (Maneb)
6 R = H; M = mixture of Zn, Cu, Mn, Fe (Cufram)
7 R = CH_3; M = Zn (Propineb)

1–7 are typical representatives of ABDTCs, **3–7** being the most frequently used fungicides in agricultural practice.

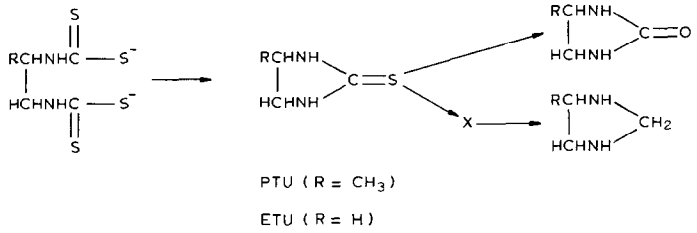
The methods developed for their determination, mainly volumetric, have low selectivity[5]. A number of them are indirect, based either on spectrophotometric, gas chromatographic (GC) or thin-layer chromatographic (TLC) determinations of the reaction products, liberated after reduction (in an acidic medium) by carbon disulphide[6–10], ethylenediamine or propylenediamine[11,12]. These determinations are not specific (they are not direct), and some are rather time-consuming. Some high-performance liquid chromatographic (HPLC) methods have also been described for the analysis of ABDTCs after extractive alkylation[13,14] and for alkylenethiourea after derivatization[15–17], as well as direct HPLC methods with spectrophotometric[18,19] or electrochemical[18,20] detection and a TLC method[21].

The aim of the present work was to apply HPLC for direct determination of the anionic part of these compounds (bis-dithiocarbamates) and their degradation products and metabolites (mainly alkylenethiourea) for toxicological investigations.

EXPERIMENTAL

Apparatus and chemicals

The HPLC equipment consisted of a Series 4 solvent delivery system (Perkin-Elmer, Norwalk, CT, U.S.A.), equipped with a Model 550 SE UV–VIS spectrometer (Perkin-Elmer) used as an UV detector with an 8-μl flow cell, an LC-95 UV detector



Metabolic degradation of ABDTCs according to Vogeler *et al.*[2]; X = unidentified product.

with a 4,5-μl flow cell or an electrochemical detector Model LC-4B with a glassy carbon working electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.), an injector Model 7125 with a 20-μl loop (Rheodyne, Cotati, CA, U.S.A.) and a Model R-100 recorder (Perkin-Elmer). The UV spectra were recorded on a Lambda-5 UV–VIS spectrometer (Perkin-Elmer). A precise pH meter "Radelkis", Type OP-207 (Metrimpex, Budapest, Hungary) and an ultrasonic bath Model RK-100 Sonorex (Bandelin, Berlin, F.R.G.) were used in the course of the sample and mobile phase preparation.

All solvents used, anhydrous methanol and ethanol, pentane, heptane and water, were of HPLC grade and all other chemicals, $NaClO_4$, EDTA disodium salt, dipotassium phosphate, sodium dihydrogenphosphate and ascorbic acid, were of analytical-reagent grade.

Water- and methanol-extracted zinc propylenebis(dithiocarbamate) $(Zn–PBDTC)_n$, supplied by Agria (Plovdiv, Bulgaria), Zineb (Zn–EBDTC), supplied by Bayer (F.R.G.), propylenethiourea (PTU), synthesized according to McKay and Hatton[22] and twice recrystallized from heptane–ethanol and ethylenethiourea (ETU) (2-imidazolidinethione), supplied by Aldrich (Milwaukee, WI, U.S.A.), were used as standard substances.

All solutions were degassed by helium purging, and thermostatted at ambient temperature in capped flasks.

HPLC procedures

Ion-exchange HPLC (HPIEC) was performed using a 250 mm × 4.6 mm Partisil SAX 10-μm (Whatman) column. The mobile phase was 1 g EDTA, 0.150 mol ClO_4 and 0.05 mol phosphate per litre water, pH 6.8, at a flow-rate of 2 ml/min. UV detection was used for concentrations higher than 1 μg/ml (285 nm), and electrochemical detection (ED) for the concentration range 1 ng/ml–1 μg/ml at a glassy carbon working electrode potential of 0.80 V; the reference electrode was 1 *M* Cl^-/Ag/AgCl.

Normal-phase HPLC was performed using a PE 250 mm × 4.6 mm Si-60 10-μm, a 125 mm × 4 mm LiChrosorb Si 100 5-μm, a 250 mm × 4 mm LiChrosorb CN 5-μm or a 125 mm × 4 mm LiChrosorb 5-μm RP-8 column. The mobile phase: comprised 6–8% (v/v) of methanol and ethanol in pentane at a flow-rate of 3 ml/min (overpressure 50 kPa helium in the mobile phase container to prevent flow fluctuations). UV detection was performed at 235 nm.

The hydrodynamic voltammogram of $PBDTC^{2-}$ was obtained by injection of aliquots equivalent to 500 ng $(Zn–PBDTC)_n$ in the mobile phase (see above) at a flow-rate of 2 ml/min.

All solutions of $(Zn–PBDTC)_n$ and $(Zn–EBDTC)_n$ were obtained by dissolution in the mobile phase (presence of EDTA) and adding ascorbic acid as an antioxidant in degassed water.

The determination of $(Zn–PBDTC)_n$ in air in the course of measurement of the inhalatory toxicity of $(Zn–PBDTC)_n$ (technical product) to experimental animals was carried out by aspiration of 20 l of the assay air through a 13-mm-diameter polymeric 1.5-μm polyvinyl acetate (PVA) fibre FPP-15 filter (U.S.S.R.), held in a filter holder. The latter was connected to a syringe and washed with 2.0 ml of methanol. After that the filter was removed and sonicated for 15 min in a capped sample tube with 5.0 ml of 0.1% EDTA and 0.1% ascorbic acid in degassed water. Aliquote of both solutions,

methanolic and water, were analysed by normal-phase HPLC–UV detection and HPIEC–ED respectively.

The sample preparation for determination of PTU and ETU in Zineb, $(Zn–PBDTC)_n$ and Maneb technical products involved a single extraction of an aliquot (10.0 mg) of the sample in a 25.0-ml flask with methanol (sonication is recommended). A part of the solution obtained was filtered through an 0.45-μm filter and an aliquot was analyzed by normal-phase HPLC–UV detection at 235 nm.

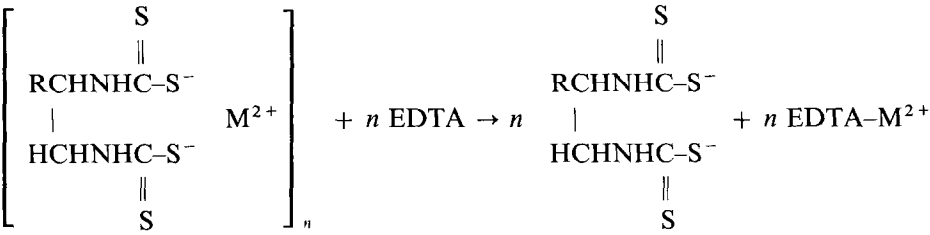
The blood samples (1 ml whole blood) were diluted in 1 ml water and 1 ml acetonitrile and then extracted with 2 ml ethyl acetate. The extract obtained was dried over magnesium sulphate and the solvent was evaporated by purging with nitrogen. The solution of the dry residue in ethyl acetate was analysed by normal-phase HPLC–UV detection at 235 nm.

The samples of rat suprarenal glands were homogenized in water–acetonitrile media. The supernatant obtained after centrifugation was extracted with ethyl acetate and the extract was dried, concentrated and analyzed as described for blood samples.

RESULTS AND DISCUSSION

Method development

The procedure for determination of ABDTCs is based on HPLC quantitation of the anionic part of the compounds (determining the biological activity of the ABDTCs) *i.e.*, on quantitation of ethylene- and propylenebis(dithiocarbamates) (see compounds **1–7**). Due to the insolubility of compounds **3–7**, a procedure for their dissolution in water based on a substitution complexation reaction[23] was used:



The presence of EDTA in the mobile phase is also necessary to prevent the accumulation of M^{2+} in the column and sedimentation of $ABDTC^{2-}$. The optimum concentration of EDTA was found to be *ca.* 1 mg/ml as at lower concentrations the dissolution of ABDTC proceeded very slowly. The same concentration was kept in the mobile phase in order to prevent the appearance of "injection peaks".

The UV spectrum of the $PBDTC^{2-}$ (that of $EBDTC^{2-}$ is similar and its change with time is presented in Fig. 1. It is evident that UV detection at 285 nm is possible for concentrations higher than 1 μg/ml (molar absorptivity, $a = 2.2 \cdot 10^4$ a.u.). The spectral changes observed indicate oxidation reactions. For that reason the use of oxygen-free water (degassed) and addition of an antioxidant (ascorbic acid) is obligatory (as confirmed by the chromatographic results presented in Fig. 2b and c).

A typical chromatogram in Fig. 2a shows the possibility of separating $EBDTC^{2-}$ and $PBDTC^{2-}$ using an ion-exchange column. The application of HPIEC–UV detection is demonstrated for quantitation of (Zn–PBDTC) in a technical product dissolved in the absence (b) and presence (c) of ascorbic acid.

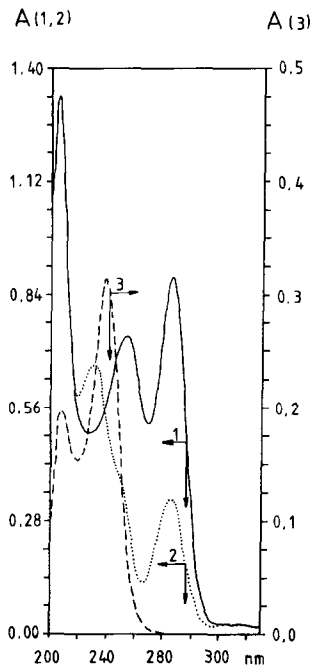


Fig. 1. UV spectra of $PBDTC^{2-}$ and PTU (optical cell length, l = 10 mm): (1) 1.2 mg $(Zn–PBDTC)_n$, dissolved in 50.0 ml degassed water in the presence of 1 mg/ml EDTA, *vs.* 1 mg/ml EDTA in water, recorded 40 min after preparation. (2) solution 1, recorded after 16 h (sealed vessel); (3) 1 μg/ml PTU solution in methanol, *vs.* methanol.

For ED to be used, the hydrodynamic voltammogram of $EBDTC^{2-}$ and $PBDTC^{2-}$ were obtained in the mobile phase as described above. The data obtained show that glassy carbon working electrode potentials above 0.6 V should be applied. The presence of EDTA in the mobile phase, however, limits the upper value of the potential, the optimum being 0.8 V.

The data presented in Fig. 3 show the optimum conditions for HPIEC determination of $PBDTC^{2-}$ using ED: pH 6.5–7 (at lower values peak broadening and tailing are observed, while values above pH 7 are harmful to the silica-based stationary phase); the retention times might be optimized by changing the perchlorate concentration; a decrease in the EDTA concentration in the mobile phase as well as the use of another complexation agent for M^{2+} with lower electrochemical activity are ways of increasing the sensitivity in HPLC–ED of $ABDTC^{2-}$. For our purposes however, the sensitivity achieved [limit of determination 100 pg injected $(Zn–PBDTC)_n$ after ionisation in EDTA solution] was satisfactory and no attempts were made to increase it.

We have demonstrated that normal-phase HPLC–UV detection is the most convenient method for determination of PTU and ETU among the well known chromatographic variants[18–20], showing a wide range of linearity (up to 2 μg injected), high efficiency and sensitivity. Both methanol and ethanol were used as polar modifiers of the mobile phase because of the impossibility of dissolving more than 6% methanol in pentane, while the use of ethanol only results in a loss of efficiency. The

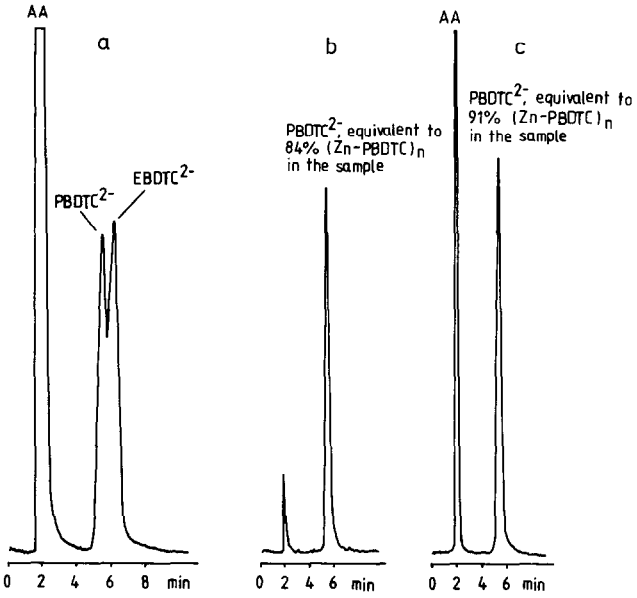


Fig. 2. Chromatograms of solutions of Zineb and $(Zn–PBDTC)_n$. Column: 250 mm × 4.6 mm SAX, 10 μm. Mobile phase: 1 g EDTA, 0.150 mol ClO_4^- and 0.05 mol phosphate per litre water, pH 6.8, flow-rate 2 ml/min. UV detection at 235 nm. (a) Equal amounts of Zineb and $(Zn–PBDTC)_n$, dissolved in the mobile phase in presence of AA; (b) 2.0 mg of $(Zn–PBDTC)_n$, dissolved in 100.0 ml of mobile phase, 10 μl injected 10 min after preparation; (c) 2.0 mg of the same $(Zn–PBDTC)_n$ as used for (a), dissolved in 100.0 ml of mobile phase containing 100 mg ascorbic acid (AA).

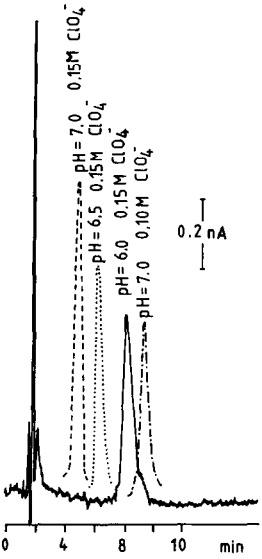


Fig. 3. HPIEC–ED of $PBDTC^{2-}$ and changes in its retention with acidity and perchlorate concentration in the mobile phase. Column: 250 mm × 4.6 mm SAX, 10 μm. The mobile phase contains 1 g/l EDTA; pH and perchlorate concentrations as shown. ECD at 0.8 V on glassy carbon working electrode (1 M Cl^-/Ag/AgCl reference electrode).

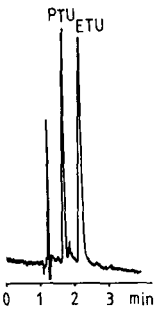


Fig. 4. Normal-phase HPLC separation of ETU and PTU. Column: 125 mm × 4 mm LiChrosorb RP-8, 5 μm. Mobile phase: 6% methanol and 6% ethanol in *n*-pentane, flow-rate 3 ml/min. Detection: UV, LC-95 (4.5-μl flow cell), 235 nm. Sample: 10 μl standard solution containing 1 ng PTU and 1 ng ETU.

typical normal-phase chromatogram obtained on a LiChrosorb RP-8 5-μm column, presented in Fig. 4, shows the possibility of separating ETU and PTU. Similar results can be obtained using Si or CN columns in the normal-phase mode. The high absorbance at 235 nm ($a = 1.0 \cdot 10^4$ a.u. for ETU and PTU) and high efficiency of

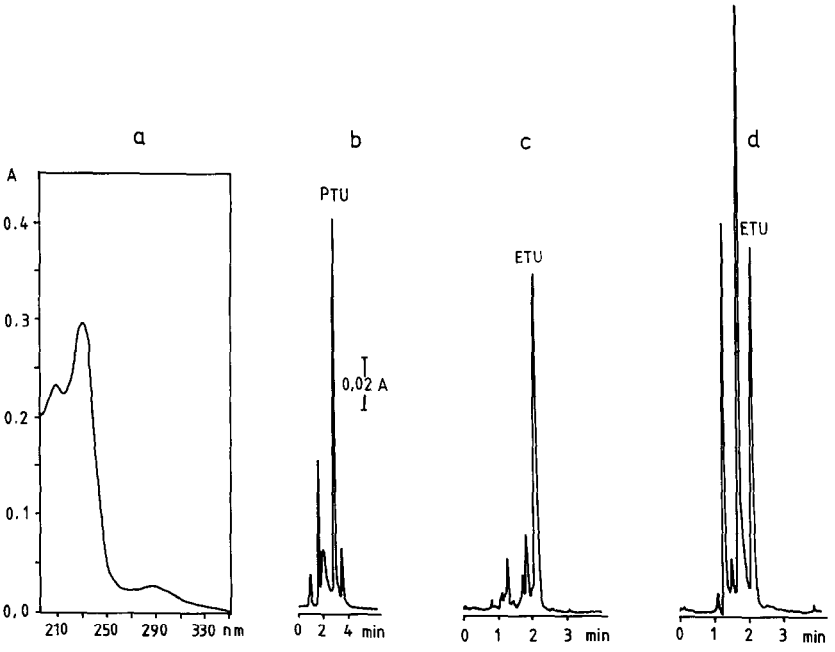


Fig. 5. Spectrophotometric and normal-phase HPLC determination of PTU and ETU in $(Zn–PBDTC)_n$, Zineb and Maneb technical products: (a) UV spectrum of a methanolic extract (25.0 ml) of 10.0 mg $(Zn–PBDTC)_n$ *vs.* methanol ($l = 10$ mm). (b) Normal-phase chromatogram of the same extract as in (a). Column: PE 250 mm × 4.6 mm Si-60, 10 μm. Mobile phase: 8% methanol and 8% ethanol in pentane, flow-rate 3 ml/min. Detection: UV, 235 nm. (c) Normal-phase chromatogram of a methanolic extract (50 ml) of 10 mg Zineb. Column: 125 mm × 4 mm LiChrosorb RP-8, 5 μm. Mobile phase 6% methanol and 6% ethanol in *n*-pentane, flow-rate 3 ml/min. Detection: UV, 235 nm. (d) Normal-phase chromatogram of a methanolic extract (50 ml) of 10 mg Maneb. Conditions as in (c).

normal-phase HPLC of the compounds allow a highly sensitive determination to be achieved (100 pg injected for PTU and ETU when an LC-95 UV detector is used).

Applications

The applications of the procedure developed for determination of PTU in a methanolic extract from a technical product $(Zn–PBDTC)_n$ is shown in Fig. 5b (the UV spectrum of the extract is shown on Fig. 5a). The appearance of several peaks in the chromatogram obtained with UV detection at 235 nm shows unequivocally that the absorbance at this wavelength is due to different compounds and selective determination of PTU is possible only by means of HPLC. In Fig. 5c and d chromatograms of methanolic extracts of Zineb and Maneb, obtained in the same way, are represented. The other peaks observed are due to impurities, formed in the course of prolonged storage of the samples (more than 4 years).

The determinations of $(Zn\text{-}PBDTC)_n$ and PTU by means of HPIEC–ED and normal-phase HPLC–UV detection, respectively, in air in the course of measurement of the inhalatory toxicity of the $(Zn–PBDTC)_n$ technical product to experimental animals are demonstrated in Fig. 6. The high reproducibility of the HPLC

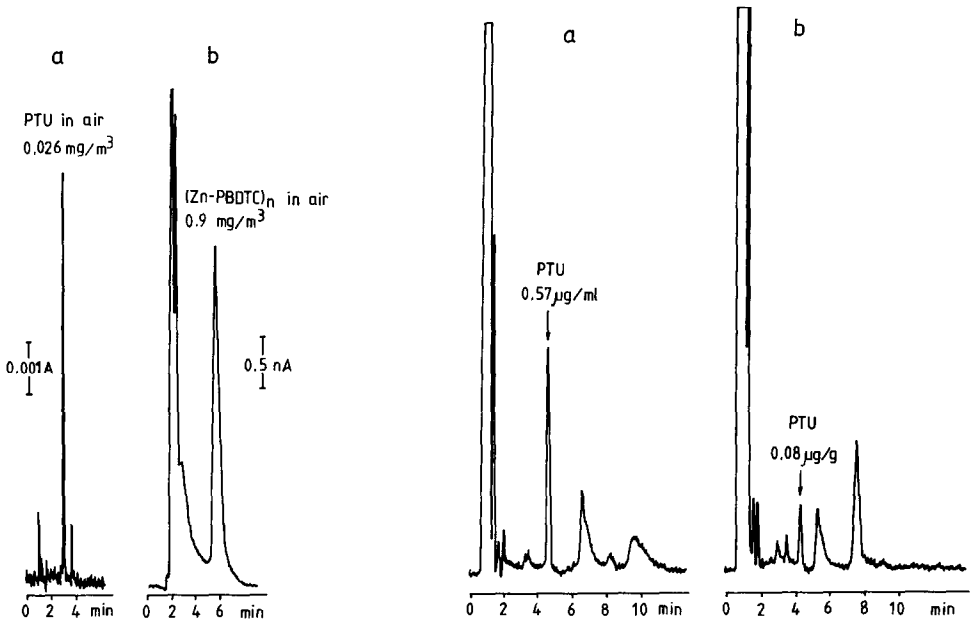


Fig. 6. Determination of $(Zn–PBDTC)_n$ and PTU in air from a camera for inhalatory experiments with experimental animals. (a) Normal-phase chromatogram of the methanolic extract from the sampling filter after aspiration of 20 l air. Conditions as in Fig. 5b. (b) Ion-exchange chromatogram of the extract of the same filter as in (a) in mobile phase in the presence of antioxidant (ascorbic acid). Conditions as in Fig. 2.

Fig. 7. Determination of PTU in biological samples. (a) Normal-phase chromatogram of a blood sample from a rat, given a dose of 60 mg/kg PTU daily for 1 month. Column: 125 mm × 4 mm LiChrosorb Si 100, 5 µm. Mobile phase: 5% methanol and 5% ethanol in pentane, flow-rate 2 ml/min. Detection: UV, 235 nm. Sample preparation as described in the text. (b) Normal-phase chromatogram of a suprarenal gland sample from a rat, dosed as in (a). Conditions as in (a). Sample preparation as described in the text.

determination was confirmed when the sample solution from a separate air sample was injected five times in 3 h [coefficient of variation C.V. = 4.6% for PTU and 8.3% for $(Zn–PBDTC)_n$]. A considerably lower reproducibility was obtained in the course of measurement of the air concentrations of PTU and $(Zn–PBDTC)_n$ [four determinations daily, C.V. 20–40% for PTU and $(Zn–PBDTC)_n$], which is connected mainly with inconsistent dosage of $(Zn–PBDTC)_n$ in the air.

Chromatograms of rat blood and rat suprarenal gland samples for determination of PTU are presented in Fig. 7. The recoveries from the procedures described are 93–97% (six determinations) for blood samples, where a known amount (5.00 μg/ml) of PTU was added, and 61–75% (three determinations) for suprarenal gland samples, where 1.0 μg PTU per gram sample was added before homogenization.

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