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THIN-LAYER CHROMATOGRAPHY OF PYRITHIONES

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SUMMARY

Pyrithione (2-pyridinethiol-1-oxide) can be quantitated as the zinc(II) complex (ZPT) by means of thin-layer chromatography. Microgram amounts of ZPT or [^{14}C]ZPT are separated from common impurities on silica gel plates by irrigation with a mixture of chloroform and methanol. Ferrous sulfate is used as a selective reagent to visualize the spot which in turn is quantitated by means of spectrodensitometry. Special conditions are used which minimize photodecomposition and chemical interaction between pyrithione and the materials in the thin-layer plate.

INTRODUCTION

This paper describes a rapid and precise thin-layer chromatographic (TLC) analysis for pyrithione (2-pyridinethiol-1-oxide), its sodium salt and its zinc complex (zinc pyrithione or ZPT) in dosing formulations for animal metabolism experiments.

Developing a repeatable and quantitative analysis is complicated by the chemical reactivity of ZPT and the free ligand PT^-. Fig. 1 illustrates some of the chemistry of PT. HPT, the free form of PT, is an acid with pK_a of 4.4 (refs. 1 and 2). Its salts, NaPT and NH_4PT, are soluble in water, methanol and to some extent in ethanol but are not soluble in less polar solvents[2]. PT^- combines with metal ions to form complexes, many of which have no net charge and are insoluble in water. Some of these complexes can be extracted into chloroform and are brightly colored[3,4]. The stability constants of PT complexes are pH-dependent and vary among different metal ions[1]. PT^- will release one type of metal ion and combine with another depending on the type and amount of ions present[1,3,5]. This kind of exchange enables determination of ZPT by formation of either Cu(II) or Fe(III) complex[6].

PT^-, its salts and its metal complexes are also light sensitive. When HPT is irradiated with ultra-violet (UV) light in any one of several organic solvents, it dimerizes[7–9]. While the extract structure of (PT_2) is uncertain (it has been identified as both 2′-dithio-bis-(pyridine-1-oxide) and 1′,4′,6′,9′-tetrahydro-1,6-diazothianthrene) it can be either reduced by further irradiation to pyridyl-2,2′-disulfide (PDS) or oxidized, presumably by reaction with molecular oxygen, to 1-oxpyridine-2-sulfonic acid (OSA).

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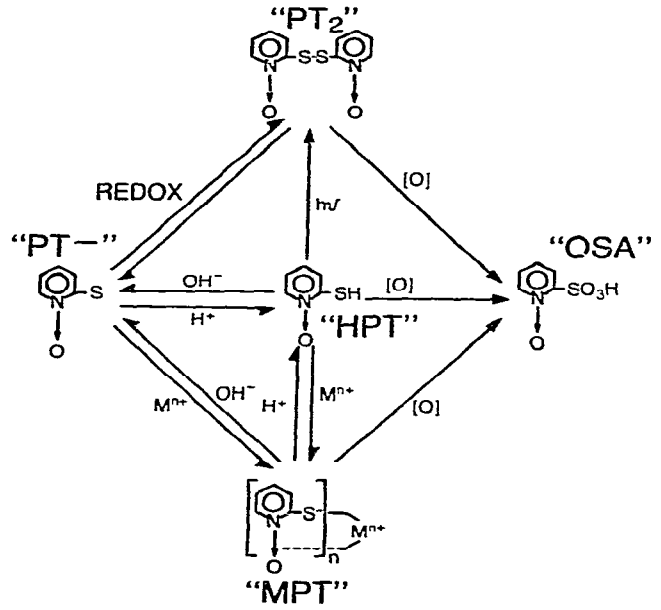

Fig. 1. Solution chemistry of pyrithione.

Separation of ZPT on TLC plates from known impurities and suspected metabolites has been reported previously[10,11]. While similar elution conditions were described in both reports, differences in elution order were reported for some compounds. In addition, both groups reported multiple spots from NaPT.

EXPERIMENTAL

Reagents

ZPT (Olin, Stamford, CT, U.S.A.; Lot No. Z07423) was found to be 97.5% pure by redox titration with iodine. Structure was confirmed by comparison of ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectra with the spectra of an authentic specimen of ZPT. Infrared (IR) spectroscopic analysis also confirmed identity.

[^{14}C]ZPT (New England Nuclear; Boston, MA, U.S.A.; Lot No. 7420144) was used for loading and light sensitivity experiments. This material assayed 96 $\pm$ 2% by reversed isotope dilution (RID). Highly purified [^{14}C]ZPT (100 $\pm$ 2% by RID) was used for extraction–recovery experiments[12].

NaPT (Olin; Lot No. 7011P220) was purified before use by means of filtration and recrystallization. Purity was determined by titration with aqueous perchloric acid to be 97% after vacuum drying for 15 h. HPT was generated from NaPT by precipitation from aqueous HCl followed by recrystallization from ethanol. Its purity was 98% by titration with aqueous NaOH. 2-Mercaptopyridine (2MP), PT_2 and OSA were obtained from Olin and were used as received.

Thin-layer chromatography

Chloroform and methanol (Burdick & Jackson Labs., Muskegon, MI, U.S.A.;

distilled-in-glass grade) were mixed 49:1 (v/v) for the eluent. Silica gel GF plates (Analtech, Newark, DE, U.S.A.; No. 2011) 250 μm, 20 × 20 cm containing 12–14% $CaSO_4$ binder and ≈4% $ZnOSiO_2$ as a phosphor were used in all studies. Plates were first washed in chloroform–methanol (2:1, v/v) for 2.5 h. They were then scored in the direction of elution to produce alternating sample and spacer lanes. The atmosphere in the chromatographic tank was kept saturated with eluent by means of a filter paper wick. Sample volumes were 10 μl or less. All spotting, transfer and irrigation were performed in the dark except where otherwise noted.

Spots were located by visualization with $FeSO_4$ (ZPT, NaPT and HPT), phosphorescence quenching (all materials) or radioscanning ([^{14}C]ZPT). Quantitation of radioactivity was by means of a radioscanner (Berthold LB 2760 scanner, BF2301 power supply, BF2305 ratemeter and Sargent-Welch XFR recorder with a Disc S-72170-50 integrator).

Loading experiment

Unlabelled ZPT (0, 1.73, 3.7, 7.3, 14.6 and 21.9 μg) was deposited in each of six lanes on a TLC plate. [^{14}C]ZPT (0.055 μg) was spotted over each dried spot. After irrigation, the percent of the radioactivity at the characteristic ZPT R_F was determined by radioscanning.

R_F values

ZPT, FePT, 2MP and PDS were dissolved in chloroform; PT_2, HPT and NaPT in methanol; and OSA in a methanol–water mixture. Of each compound 4–7 μg were chromatographed using chloroform–methanol (49:1). HPT and NaPT were also dissolved in, and irrigated with, a mixture of chloroform–methanol–formic acid (400:8:1). R_F values were measured during irradiation of the plate in a dark box with UV light at 254 nm.

$FeSO_4$ visualization

A 4–7-μg amount of each of ZPT, FePT, 2MP, PDS, PT_2, HPT and NaPT was spotted on a TLC plate. When dry, the plate was sprayed with a freshly prepared aqueous solution of 0.09 *M* $FeSO_4$ in water and allowed to stand one hour. In a separate experiment, ZPT was dissolved in chloroform and 1.1, 2.2, 3.3, 4.4, 5.4, 6.4, 7.5, 8.6 and 9.6 μg were spotted in separate lanes of a TLC plate. The plates were irrigated, dried, sprayed with $FeSO_4$ solution and allowed to stand for one hour. The optical densities of the resulting black spots were measured by transmission spectrodensitometry at 535 nm (Schoeffel Model SD3000, Model 300 Density computer and Varian CDS 111 integrator).

Light sensitivity

Four sunlamps (Westinghouse, 40 W) were suspended 51 cm above two TLC plates. Spacing of the lamps 25 cm apart in a plane parallel to that of the plates provided uniform irradiation. A 10.6-μg amount of [^{14}C]ZPT was spotted in the dark at the origin of each of five lanes on each plate. An opaque shield was placed over the plates exposing only one ZPT spot when irradiation began. Every five minutes thereafter the shield was moved to expose another lane. After twenty minutes, the time interval was increased to ten minutes. After sixty minutes, fresh [^{14}C]ZPT was spotted

in the sixth lane of each plate and both plates were immediately irrigated in the dark. The plates were then radioscanned to locate all spots and to determine the ^{14}C distribution among them.

Potentiometric determination of the chloroform–water partition coefficient

The titration of pyrithione, described above, was repeated except that an equal volume of chloroform was added to the titration flask (100 ml $CHCl_3$–100 ml 0.005 *M* $NaClO_4$). The 0.100 *M* NaOH was then added in increments to the stirred mixture. After each addition, the phases were allowed to separate and the pH of the aqueous phase was measured. The exact pH at the half-equivalence point was interpolated graphically.

Photometric determination of the chloroform–water partition coefficient

The absorbence (*vs.* chloroform) of a 0.015 *M* solution of HPT in $CHCl_3$ was measured at 284 nm (Cary Model 118 spectrophotometer) before and after equilibration with an equal volume of an aqueous solution of 0.005 *M* $NaClO_4$ at pH 6.35, 7.10 and 9.00, respectively. pH was adjusted by addition of 0.1 *M* NaOH.

Extraction of ^{14}C from saline

Saline solution containing 10 μg/ml [^{14}C]ZPT was processed in three different ways. (1) A 10-μl aliquot was deposited at the origin over 7.5 μg of untagged ZPT. (2) A 1.0-ml volume of solution was freeze-dried in a 50-ml round bottom flask. The residue was dissolved in 1.0 ml chloroform and 10 μl of this solution was spotted over 7.5 μg of untagged ZPT on a TLC plate. (3) A 1.0-ml volume of solution was freeze-dried and taken up in a chloroform solution containing 1 mg/ml of untagged ZPT; 10 μl was spotted for analysis. Samples were then irrigated in the dark and the ^{14}C distribution on the TLC plate was determined by radioscanning.

The same saline solution was adjusted to pH 7.2 and equilibrated with an equal volume of chloroform. A 10-μl aliquot of the chloroform layer was analyzed by TLC–radioscanning.

Repeatability

An aqueous solution known to originally contain 10 μg/ml [^{14}C]ZPT (RID) was allowed to stand in a glass stoppered container for 18 months with no special protection from either light or air. Six 1.0-ml aliquots were individually freeze-dried and taken up in an equal volume of chloroform containing 1 mg/ml of untagged ZPT. A 10-μl volume from each work-up was chromatographed and radioscanned. The R_F values and the percentage ^{14}C in each of the resulting spots in each sample lane were measured.

RESULTS

Separation of pyrithiones

The elution behavior of the test compounds is shown in Table I.

Only PDS has an R_F near that of ZPT, and while not found in any of our studies as an impurity of ZPT, it can be distinguished from ZPT by its failure to give a positive color test with $FeSO_4$ spray reagent.

TABLE I

DISTRIBUTION OF MATERIALS AFTER IRRIGATION IN CHLOROFORM–METHANOL 49:1 (v/v)

Compound spotted	R_F								
	0	*0.07*	*0.15*	*0.27*	*0.35*	*0.40*	*0.45*	*0.50*	*0 55*
ZPT									All
FePT						All			
NaPT		Trace		Most				Trace	
HPT		Trace			Most				Trace
HPT*							All		
PDS									All
PT_2		All							
2MP			All						
OSA	All								

* Chloroform–methanol–formic acid (400:8:1, v/v/v).

Elution order is dependent on the "polarity" of the molecules; acids and bases (OSA and 2MP) adhere strongly to silica gel and have low R_F values whereas neutral complexes (ZPT and FePT) have high R_F values.

NaPT and HPT each give three UV absorbing spots when separated. 5–10% of the material is at R_F 0.07 corresponding to PT_2 (dimer formation). The rest is distributed between an R_F of 0.26 (NaPT) or 0.35 (HPT) and an R_F close to that of ZPT. The formation of ZPT is explained by the presence of the zinc silicate phosphor in the TLC plates. To see if the formation of multiple spots could be prevented by preventing dissociation of HPT, 0.25% formic acid was added to the eluent. This time HPT behaved differently, giving a single spot, R_F 0.44. However, the spot was black, suggesting that HPT and trace iron in the plate had combined to form the iron complex.

Loading phenomena

During elution of ZPT, a small amount sorbs to the origin and is deposited in the path of the eluting spot. More than 3 μg must be spotted to ensure that $\geqslant$98% ZPT reaches its characteristic R_F (0.53) (Fig. 2). Fig. 3 shows the effect of mass on recovery. If small amounts of [^{14}C]ZPT must be separated, untagged ZPT may be overspotted at the origin prior to elution so that the total mass is $\geqslant$3 μg. When this is done, ^{14}C tagged and untagged material equilibrate. If the molar excess of cold ZPT is large (100 fold), then either [^{14}C]HPT, or [^{14}C]NaPT will behave just like [^{14}C]ZPT, to which they are converted. During TLC, 99% of the radioactivity will move to the characteristic R_F for ZPT. TLC methods using this overspotting technique will therefore measure all exchangeable forms of [^{14}C]PT^- as [^{14}C]ZPT.

Light sensitivity

ZPT decomposes in sunlight on TLC plates. It will not decompose, during the course of an analysis, when protected from light. Fig. 4 shows the normalized distribution of radioactivity among ZPT and two photodecomposition products "B" and "C" during exposure to artificial sunlight.

ZPT is converted to B ($R_F \approx 0.1$) which in turn goes to C ($R_F \approx 0$). The rate

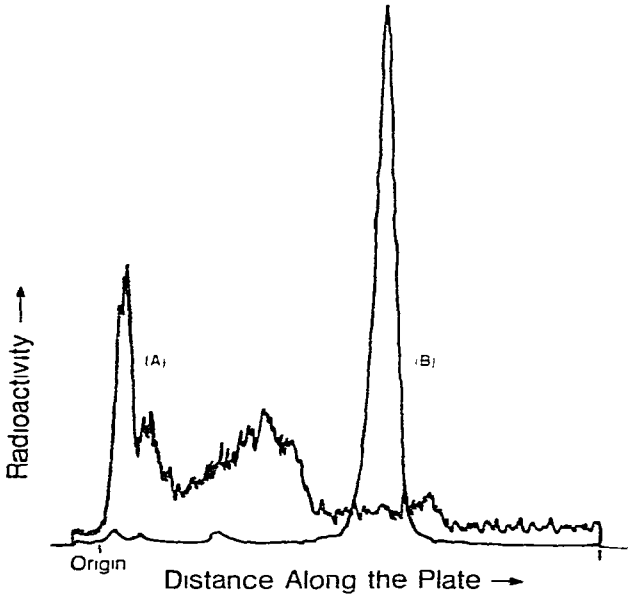

Fig. 2. Radioscan of the TLC plate after elution of (A) 0.55 μg [^{14}C]ZPT and (B) 14.6 μg [^{14}C]ZPT.

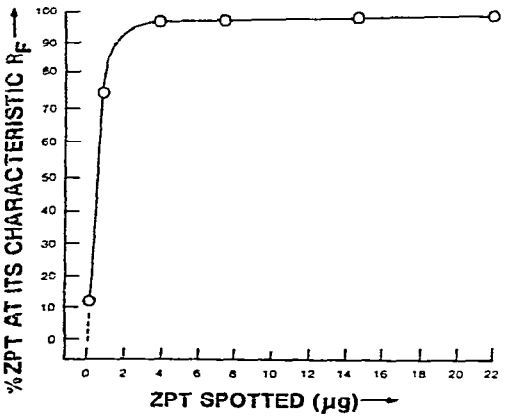

Fig. 3. "Threshold" effect for elution of ZPT.

at which ZPT disappears depends on the amount of ZPT on the TLC plate. Disappearance rate can be expressed,

$$-\frac{\text{d (mass ZPT)}}{\text{d (time)}} = K\,(\text{mass ZPT})^2$$

K, under these conditions, is $1.3 \cdot 10^{-2}\ \text{g}^{-1}\ \text{min}^{-1}$. This means only about 10% of a typical ZPT sample will remain unchanged after one hour.

The identities of B and C are uncertain although previous literature and R_F values for model compounds suggest that ZPT goes to PT_2 and PT_2 in turn to OSA. The "2nd order" dependence of [^{14}C]PT is consistent with such a mechanism.

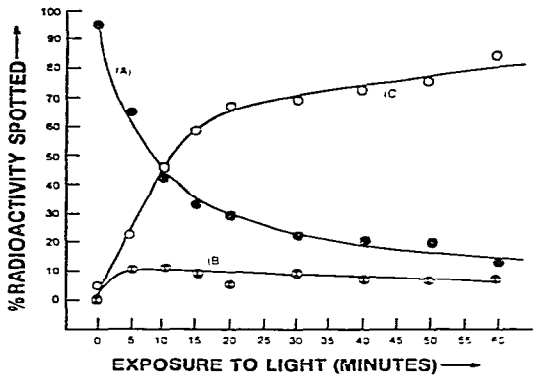

Fig. 4. The decomposition of spot A (ZPT) to spot B and in turn to spot C with exposure to artificial sunlight and air on TLC plates.

Visualization of pyrithiones

ZPT, NaPT and HPT are visualized by spraying with an aqueous solution of $FeSO_4$. The purple-black Fe(III) complex is formed on the TLC plate as Zn(II) is displaced and Fe(II) is oxidized[4]. A typical single beam densitometer trace is shown in Fig. 5. The optical density of spots containing from 1–10 μg ZPT visualized in this fashion varies linearly with the amount of ZPT in the spot. This reagent is selective for chemicals with "exchangeable" or "available" PT. It will not give a colored product with PT_2, PDS, or OSA. 2MP, which also forms a complex with iron, gives only a faint spot when sprayed.

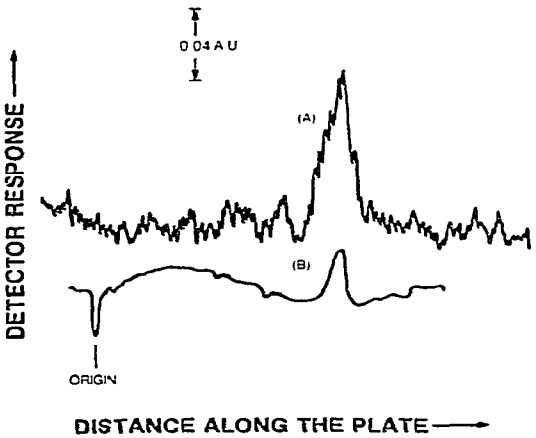

Fig. 5. The detection of 7 μg [^{14}C]ZPT after conversion to the iron complex by (A) radioscanning and (B) transmission spectrodensitometry.

Preparation of aqueous solutions for analysis

Because ZPT is not very soluble in water ($\approx 3 \cdot 10^{-5}$ M) aqueous solutions are necessarily dilute. [^{14}C]ZPT, however, can be recovered from aqueous solutions (saline solutions or water solutions with a lower salt content) and concentrated in

two steps. The salt solution is first freeze-dried to remove water. The residue is then extracted with chloroform containing 1 mg/ml untagged ZPT carrier. Using this procedure, 99.4% of the ^{14}C is taken up by the chloroform solution with one equilibration and is ready for the TLC analysis. Neither direct liquid–liquid extraction (chloroform–saline) nor extraction of the freeze-dried residue without a cold carrier will give complete recovery of [^{14}C]ZPT from water or saline. Extraction from saline without addition of extra untagged ZPT gives erratic results with ^{14}C recoveries ranging from 86 to 95%.

HPT, on the other hand, can be quantitatively removed from acidic aqueous solutions (pH 3 or below) by a single equilibration with an equal volume of chloroform. HPT partition between chloroform and water can be described mathematically:

$$\% \text{ PT in } CHCl_3 = \frac{K_p}{K_p + 1 + K_a/[H^+]} 100\%$$

K_p is the chloroform–water partition coefficient for HPT and K_a is its acid dissociation constant. This is based on a simple partition model for monoprotic acids which assumes that: (1) only HPT is extracted into the organic layer, (2) there is no dimerization in either phase, and (3) the only meaningful forms of pyrithione in the aqueous phase are HPT and PT^-. K_a was determined to be $(2.88 \pm 0.2)\ 10^{-5}$ by potentiometric titration at an ionic strength of 0.0005. K_p was found to be 68 based on the average of results from two different experiments. When HPT is titrated in water containing an equal volume of chloroform the pH of the aqueous layer at the half-equivalence point is shifted to a higher pH than when HPT is titrated in water alone. This pH shift can be used to calculate K_p using the following expression:

$$pH_{1/2} - pK_a = \log(K_p + 1)$$

K_p was calculated to be 63 by this method.

K_p was also determined spectrophotometrically. K_p was calculated using the absorbance at 284 nm of the chloroform layer before and after equilibration with an equal volume of water (0.005 M $NaClO_4$). K_p values of 71, 70 and 73 were obtained at pH 6.35, 7.10 and 9.00, respectively, with an expression derived from the definition of K_a, K_p and Beer's law:

$$K_p = \frac{A}{A_i - A}\left(1 + \frac{K_a}{[H^+]}\right)$$

A_i and A represent the absorbance of the chloroform layer before and after equilibration with the aqueous phase respectively.

Repeatability

The results of the analysis of an aged aqueous solution of [^{14}C]ZPT are shown in Table II. It is clear that the purity of [^{14}C]ZPT can be determined by TLC. R_F values of the eluted spots suggest some dissociation of ZPT as well as degradation to PT_2 and finally OSA.

TABLE II

RESULTS FRON REPETITIVE ANALYSIS OF "AGED" AQUEOUS ZPT SOLUTION

Sample No.	*Spot 1 (%)*	*Spot 2 (%)*	*Spot 3 (%)*	*ZPT (%)*	*R_F of ZPT*
1	24.6	3.7	ND	71.3	0.55
2	23.9	2.6	1.9	71.2	0.54
3	24.3	2.3	2.0	71.4	0.55
4	25.1	2.3	2.4	69.9	0.55
5	23.6	4.1	2.4	70.0	0.57
6	28.9	2.6	ND	68.9	0.58
Mean	25.1	2.9	1.5	70.5	0.56
R.S.D.	2.0	0.8	1.1	1.0	2.6

CONCLUSIONS

The purity and identity of ZPT can be repeatably and accurately determined by TLC if precautions are taken in sample preparation and chromatographic analysis. Because small amounts of ZPT will irreversibly sorb to silica gel plates, more than 3 μg must be spotted for quantitative migration of the spot. Smaller masses of [^{14}C]HPT may be overspotted with untagged ZPT for quantitative elution and then radioscanned for [^{14}C] content. Because [^{14}C]HPT and [^{14}C]NaPT both are changed to [^{14}C]ZPT when overspotted with excess untagged ZPT, overspotting methods measure total [^{14}C]PT. This technique, therefore, measures PT^- in any exchangeable form.

ZPT will decompose on TLC plates unless protected from light. The TLC behavior of the resulting decomposition products suggest dimerization followed by oxidation to the sulfonic acid. Similar changes can occur when ZPT is dissolved in saline and allowed to stand. This is consistent with previous work[7–9,13].

NaPT and HPT are difficult to quantitate by means of TLC because they react with metals in the TLC plate. In acidic eluents, the iron complex is formed. "Neutral" eluents yield ZPT from reaction with the phosphor. This reactivity with metals, however, can be used to advantage in analyzing for either HPT or NaPT by TLC. With proper control of pH and concentration, the Zn(II) or Fe(III) complexes are easily formed and are isolated for analysis by extraction[4,6]. The concentration of pyrithione in this the resulting TLC spot may be determined by fluorescence quenching or transmission densitometry.

Dilute aqueous solutions of ZPT can be concentrated and taken up in chloroform by first freeze-drying the solution followed by extraction with a carrier (excess ZPT). This is consistent with solvent extraction studies conducted by Hartman and Davis[14]. They monitored $[^{65}Zn]^{2+}$ and [^{14}C]PT^- and found that at pH 7.4 stoichiometric extraction of ZPT was maximum (but not quantitative) from aqueous solution into chloroform. The Zn(II) extraction further increased at higher pH while PT extraction was less than 2 to 1 (PT to Zn). This suggests that ZPT dissolves in water through partial dissociation to mixed complexes containing both PT^- and OH^-. This dissociation is reversed or overridden by removal of water and equilibration with excess ZPT in chloroform.

The data presented here when combined with Hartman and Davis' work on

ZPT extraction suggests that excess ligand can be quantitatively (99%) removed from a saturated solution of ZPT in chloroform by equilibration with an equal volume water at ≈pH 8. This technique could be used to separate these species prior to analysis of each individually.

Finally, the TLC behavior of pyrithione suggests another explanation for literature reports of TLC (or paper chromatography) spots with R_F values similar to those of PT_2 or OSA in work-ups of specimens from animals that were dosed with either NaPT or ZPT[10,11,15,16]. Without special precautions in preparation of the dosage media, processing of the specimens and TLC analysis one would surely get PT_2 and OSA from photodimerization or oxidation and ZPT from complexation with $ZnOSiO_2$ in the TLC plate even if no biotransformation occurred.

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