Use of Paper Partition and Thin-Layer Chromatography for Identification of Active Ingredient in Dursban Insecticide and Its Possible Metabolites

GRANT N. SMITH AND FAYE S. FISCHER

Paper partition and thin-layer chromatographic procedures have been developed for the isolation and identification of Dursban and its breakdown products. These procedures have been used to identify the products formed by biological and chemical breakdown of the Dursban.

The paper-partition chromatographic procedures developed by Consden *et al.* (*1*) have been found useful in the separation and identification of many classes of compounds (*1–7, 15*). Similarly, thin-layer chromatography techniques have received a considerable amount of attention in recent years for the separation of compounds of biological interest.

During a series of recent investigations on the metabolism of Dursban insecticide, it was found desirable to adapt both the paper chromatography and thin-layer chromatography techniques for the separation and identification of *O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate (found in Dursban insecticide, and which will hereafter be referred to as Dursban, for purposes of brevity), its related decomposition products, and related pyridinols (*11–14*).

To adapt these techniques to the study of Dursban and related compounds, it was necessary to develop colorimetric procedures which could be used to locate the positions of the compounds on the paper strips or thin-layer chromatograms. A review of the various structures which might be encountered indicated that no single test could be used to identify all the compounds involved.

A study of the chemistry of the Dursban molecule indicated that the compound could undergo hydrolysis with the formation of a series of phosphorothioic acids. The sulfur could also be replaced with oxygen, in which case a series of phosphoric acids would be obtained. Once the pyridinol moiety has been split from the phosphorothioate or phosphate, it can undergo dehalogenation with the formation of a series of diols and triols. These compounds appear to be very unstable and undergo cleavage of the ring with the formation of various low molecular weight fragments, such as carbon dioxide.

By the use of specific chemical tests, Dursban and most of its decomposition products can be identified and separated.

Biomechanisms Laboratory, E. C. Britton Laboratory, The Dow Chemical Co., Midland, Mich.

Methods

The paper-partition chromatography was carried out with either Whatman No. 1 filter paper strips 2 × 50 cm., or sheets 45 × 55 cm. using the descending method (*1*). Approximately 0.05 ml. of the solution containing about 20 μg. of the compound was applied 8 cm. from one end of the filter paper in such a manner that the area covered by the solvent did not exceed 0.5 cm. in diameter.

After being air-dried, the paper was developed at 25° C. in an all-glass chamber shown in Figure 1. The chamber had been allowed to stand overnight with the solvent system to ensure its saturation with the vapors of the solvent system.

The strips containing the unknown samples together with strips containing the reference standards were introduced into the chamber. The jar then was closed and the solvent system introduced through the hole

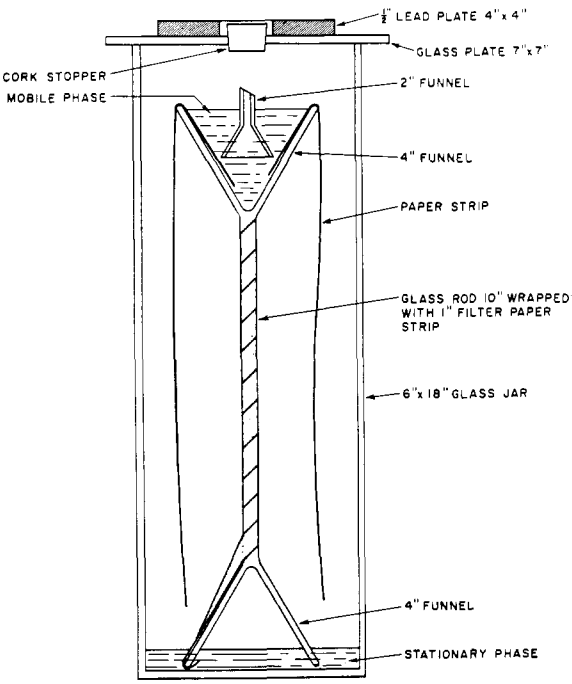


Figure 1. Chromatographic jar

in the glass plate on the top of the jar. The chromatograms were developed in the dark to prevent light-induced decomposition of the compounds. In working with these compounds, great care must be exercised in preventing them from being exposed to light, especially ultraviolet or sunlight, during the time they are in solution. If the chromatograms are allowed to stand overnight under light, many of the derivatives of the 3,5,6-trichloro-2-pyridinols will undergo oxidation.

After the solvent had run the desired distance (25 to 30 cm.), the strips were removed, the solvent front was marked, and the paper was dried at room temperature in the dark. The compound should be detected on the strips as soon as possible since some of the compounds are volatile and may disappear from the strips on long standing.

In those cases where radioactive labeled compounds were available, they were used to establish the R_f values of these compounds. In the case of the paper strip chromatograms, the position of the radioactive compounds were determined by scanning the strips in a Nuclear-Chicago Actigraph III or by exposing them to No-Screen x-ray film to form radioautographs. In the case of the paper sheets or thin-layer chromatograms, radioautographs were made to detect the location of the radioactive compounds.

A series of chemical tests was used, depending on the nature of the compound.

The Hanes-Isherwood test was used to detect those compounds containing a phosphate or phosphorothioate group. The test was run by spraying the strips with a fresh solution made by mixing 95 ml. of 1% ammonium molybdate in 0.1*N* HCl with 5 ml. of 60% perchloric acid. The strips were air-dried until the excess water was removed, and the strips were then placed in a Chromato-Vue box and exposed to ultraviolet light for 15 to 20 minutes. Most of the compounds would give a blue color under these conditions. If not, the strips were exposed to H_2S gas to intensify the spots further.

In some cases, the compounds could not be hydrolyzed by exposing them to ultraviolet light. Under these conditions, the strips first were sprayed with Hanes-Isherwood reagents and then heated for 10 minutes in an autoclave at 120° C. The strips then were exposed to the ultraviolet light according to the standard procedure.

Those compounds containing the pyridinol group can be detected by treatment with bromine followed with starch-KI. In this procedure, the air-dried strips were heated for 5 minutes at 75° C. to remove all traces of the solvent. The strips then were exposed to bromine vapors for about 30 seconds, and aerated until the excess bromine had been removed. This can be determined easily by treating a section of the chromatogram with the starch-KI spray solution. If the excess bromine has been removed, the strip will give no significant color with the starch-KI solution. In practice, a Q-tip is used to apply a small quantity of the starch-KI solution to the strip above the origin. This operation can be repeated several times without injuring the chromatogram. The starch-KI solution contains 0.5% starch and 0.5% potassium iodide. When the strips are sprayed with the starch-KI solution, the spots will appear as dark blue areas with a light blue or colorless background.

The pyridinol compounds also can be detected by spraying the air-dried strips with a freshly prepared solution containing 20 ml. of a 1% solution of ferric chloride, 60 ml. of water, and 20 ml. of a 1% solution of potassium ferricyanide. The spots are blue against a yellow background.

The partially oxidized products of the pyridinol can be detected by spraying the strips with 0.1*N* sodium hydroxide or exposing the strips to H_2S gas. With thin-layer plates, it was difficult to obtain good color tests with all the compounds. However, the new Eastman chromatogram sheets containing silica gel and lead-manganese-activated calcium silicate as a fluorescent indicator could be used very satisfactorily without the necessity of using a color development step.

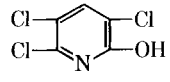
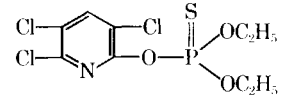
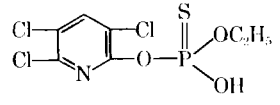
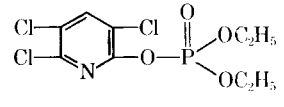
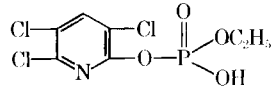
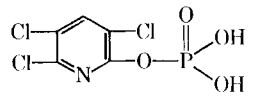
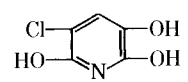
Proper precautions should be used in handling perchloric acid, hydrogen sulfide, and bromine vapors used in these chromatographic studies. Investigators should refer to the National Safety Council Industrial Data Sheets D311, 284, and 313.

With the TLC methods, the sheets were developed in the special chromatographic unit designed to be used with this film. After development with the chromatographic solvents, the sheets were air-dried and viewed

Table I. Chromatographic Systems Used for the Separation and Identification of Dursban and Its Possible Metabolites Using Paper Partition Chromatography

System Number	Composition
E 1	22% *tert*-Butyl alcohol
	9% Glacial acetic acid
	69% Water
E 2	30% Ethyl alcohol
	30% *n*-Propyl alcohol
	5% Concd. ammonium hydroxide
	35% Water
E 3	40% Ethyl alcohol
	40% *n*-Propyl alcohol
	5% Concd. ammonium hydroxide
	15% Water
E 4	75% Isopropyl alcohol
	2% Concd. ammonium hydroxide
	23% Water
E 5	80% Acetonitrile
	2% Concd. ammonium hydroxide
	18% Water
E 6	50% Glacial acetic acid
	50% Benzene
135	10% *n*-Propyl alcohol
	2% Concd. ammonium hydroxide
	88% Water
141	90% *n*-Propyl alcohol
	2% Concd. ammonium hydroxide
	8% Water

Table II. R_f Values of Dursban and Related Compounds with Various Chromatographic Systems

Name	Structure	Paper Chromatography Solvent Systems								Thin-Layer Solvent Systems		
		E-1	E-2	E-3	E-4	E-5	E-6	135	141	TL-1	TL-2	TL-3
3,5,6-Trichloro-2-pyridinol		0.80	0.90	0.76	0.78	0.68	0.94	0.78	0.62	0.20	0.17	0.75
O,O-Diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate		0.92	0.96	0.94	0.94	0.98	0.94	0.00	0.94	0.93	0.72	0.92
O-Ethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate		0.86	0.92	0.76	0.78	0.78	0.84	0.86	0.70	0.44	0.00	0.82
Diethyl 3,5,6-trichloro-2-pyridyl phosphate		0.80	0.88	0.90	0.90	0.95	0.90	0.00	0.92	0.87	0.34	0.90
Ethyl 3,5,6-trichloro-2-pyridyl phosphate		0.88	0.90	0.75	0.79	0.60	0.95	0.87	0.67	0.14	0.00	0.10
3,5,6-Trichloro-2-pyridyl phosphate		0.82	0.85	0.59	0.59	0 48	0.07	0.96	0.00	0.20	0.00	0.31
Sodium chloride	NaCl	0.78	0.69	0.38	0.35	0.29	0.00	0.90	0.08	0.00	0.00	0.00
Sodium bicarbonate	$NaHCO_3$	0.00	...	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5-Chloro-2,3,6-(1H) pyridine-trione		0.72	0.00	0.00	0.00	0.00	0.64	0.84	0.15	0.00	0.00	0.00

in the Chromato-Vue box using the short wavelength ultraviolet light (240 mμ). The radioactive compounds then could be located by exposing the chromatogram sheets to No-Screen x-ray film.

In preparing the chromatogram sheets for exposure, the positions of the nonradioactive compounds were outlined with radioactive ink so that they would be shown on the radioautograph. The data concerning the nature of the samples, etc., also were stamped on the sheets so that it would be printed on the radioautograph.

Results and Discussion

In Table I are listed the various solvent systems used in the paper chromatographic techniques. The R_f values obtained with these systems using Dursban and related compounds are shown in Table II. The R_f values listed are the average of 10 or more runs using authentic samples of the compounds.

The structures of the individual compounds were established by the method of synthesis and chemical and physical analysis. These samples were analyzed by infrared, mass spectroscopic, and NMR analysis. Elemental analyses for carbon, hydrogen, oxygen, sulfur, phosphorus, and nitrogen also were made to aid in the establishment of the structure. Many of these compounds were susceptible to hydrolysis, dehalogenation, and oxidation when they were in solution exposed to the light. Under these circumstances, the original compounds were stored in brown bottles in the refrigerator, and fresh solutions were made for each set of chromatograms.

The variations observed in the R_f values of the same compound in repeated identical runs were less than ±5%. The solvents used were all redistilled. However, there were variations in the R_f values when the same solvent was obtained from various sources. This was especially true with the acetonitrile.

In all of the biological investigations conducted with Dursban in which chromatographic procedures were to be used (*8*, *10–14*) it was found advisable to obtain sufficient quantities of each solvent to last throughout the individual experiment.

Reference standards always were run with each set of chromatograms as a basis of comparison.

Because of the many factors influencing the R_f values of the compounds, great significance should not be placed upon their absolute magnitude. More importance should be given to the relative movement of the various compounds in relation to the movement of the reference standards.

When biological extracts were being studied, the reference compounds were added to aliquots of the extract before they were spotted on the strips or plates so that all compounds would be chromatographed under the same conditions. Whenever possible the radioactive compounds were used. The radioactive compounds had high enough specific activities (1.5 to 3 mc. per mmole) that they could be added to the biological material and could be detected easily by the Nuclear-Chicago Actigraph III, but would be present in such small quantities that they would not be detected by the chemical sprays. This had a distinct advantage in establishing whether the authentic compound was superimposable on the unknown compound.

Table III. Chromatographic Systems Used for the Separation and Identification of Dursban and Its Possible Metabolites Using TLC Methods

System Number	Composition
TL-1	70% Acetonitrile
	10% Hexane
	15% Acetone
	5% Concd. ammonium hydroxide
TL-2	80% Hexane
	18% Acetone
	2% Concd. ammonium hydroxide
TL-3	93% Acetone
	3% Methyl alcohol
	2% Concd. ammonium hydroxide

The solvent system used for most of the paper chromatographic investigation was system E 5. This system has the advantage that Dursban and its primary hydrolysis products in which the phosphorus was still present have R_f values greater than 0.50. The decomposition products of the 3,5,6-trichloro-2-pyridinol have R_f values below 0.50. When the 3,5,6-trichloro-2-pyridinol undergoes dehalogenation, diols and triols are formed which are water-soluble and very unstable (*8*, *10*). By using solvent system E 5 the unknown compounds can be divided roughly into two classes—the decomposition products of the 3,5,6-trichloro-2-pyridinol and the primary hydrolysis products of the Dursban.

The solvent systems used with the thin-layer chromatography are shown in Table III and R_f values of the compounds are shown in Table II.

In practice, solvent system TL-1 was used to separate the hydrolysis products of Dursban while system TL-2 was used to separate the *O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate from the diethyl 3,5,6-trichloro-2-pyridyl phosphate.

In most insect metabolism studies with phosphorothioate insecticides, there is a great deal of interest in determining if the $-P(=S)<$ compound can be converted to the $-P(=O)<$ compound without the removal of the other groups attached to the phosphorus. In many cases it is known that the $-P(=O)<$ compound is the active compound. The $-P(=O)<$ compounds are generally more potent cholinesterase inhibitors than are the $-P(=S)<$ compounds.

System TL-2 can be used easily to separate the $-P(=S)<$

compound from the diethyl 3,5,6-trichloro-2-pyridyl phosphate (the —P(=O)< compound).

The use of the Eastman chromatogram sheets greatly simplifies the identification of compounds related to Dursban. It is not necessary to activate the film by heating 15 minutes at 100° C. prior to use. The film can be spotted, developed, and viewed within 1 hour.

Literature Cited

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