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PUBLISHED PAPER'S
Thin-Layer Chromatographic Detection of Organophosphorus Insecticides Containing a Nitrophenyl Group

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A Griess reaction that has been used to detect organic compounds containing an aromatic amino group was used to detect organophosphorus compounds containing a nitrophenyl group, such as ethyl parathion, methyl parathion, and fenitrothion. On reduction with stannous chloride in HCl–water (1 + 1), these compounds give respective amino derivatives, which are further diazotized and coupled with 1-naphthylamine to give intense pink-orange spots. This reagent also gives a bluish to violet spot with p-nitrophenol, a metabolite of fenitrothion. The procedure can be used to detect these insecticides in biological materials in forensic toxicology.

Organophosphorus insecticides containing an aromatic nitro group (–NO₂) in their molecules, such as ethyl parathion, methyl parathion, and fenitrothion, are widely used in agriculture. They are frequently misused in homicidal and suicidal poisoning cases, and their characterization is, therefore, necessary. Thin-layer chromatography (TLC) is the method of choice for the identification of these insecticides in biological material.

Reagents that can be used to detect organophosphorus insecticides by TLC include palladium(II) chloride (1, 2), 4-(p-nitrobenzyl)pyridine tetraethylene pentamine (3), bromine fluorescein silver nitrate (4, 5), congo red (6), mercury(I) nitrate (7), mercury(II) nitrate–potassium hexacyanoferrate(II) (8), and acidified potassium iodide–starch (9). Hamada (10) reported the detection of parathion from organs of cadavers, and Gage (11) used KOH solution to detect parathion and related nitrophenyl derivatives by paper chromatography.

In this paper, we describe the use of the Griess reaction to detect organophosphorus insecticides containing a nitrophenyl group by TLC. After reduction with acidic stannous chloride, these insecticides give respective amino derivatives, which are further diazotized and coupled with 1-naphthylamine. This reagent gives an intense pink–orange spot with organophosphorus insecticides containing nitrophenyl groups.

Experimental

Materials

All reagents were analytical reagent grade. Distilled water was used throughout.

(a) Silica gel.—Silica gel G; particle size, 15 µm (E. Merck, Darmstadt, Germany).

(b) Insecticide standard solutions.—Technical-grade ethyl parathion, methyl parathion, and fenitrothion were used as standards. Prepare a 1 mg/mL solution in ethanol of each insecticide.

(c) Stannous chloride solution (5%).—Dissolve 5 g stannous chloride in 100 mL 50% (v/v) HCl (32%) in distilled water.

(d) Sodium nitrite solution (5%).—Dissolve 5 g sodium nitrite in 100 mL 10% (v/v) acetic acid solution.

(e) 1-Naphthylamine reagent (0.1%).—Dissolve 0.1 g 1-naphthylamine in 10 mL glacial acetic acid and dilute to 100 mL with distilled water.

Extraction of Insecticides from Biological Material

Approximately 50 g portions each of various visceral tissue types (stomach, intestine, liver, spleen, and kidney) containing insecticide were minced in aqueous solution. The insecticide was extracted with diethyl ether, and the solvent was evaporated at room temperature. The residue was dissolved in 1–2 mL ethanol. A known volume of the solution was spotted on an activated TLC plate together with the standard solutions of the insecticides.

TLC Procedure

A standard glass TLC plate was coated with a slurry of silica gel G in water (1 + 2) to a thickness of 0.25 mm. The plate was activated by heating at 110°C for ca 1 h. Standard solutions (10 µL) of ethyl parathion, methyl parathion, and fenitrothion (Sumithion) in ethanol (each at a concentration of 1 mg/mL) were spotted on the plate, which was then developed in a previously saturated TLC chamber with hexane–acetone (9 + 1) as solvent. After the solvent had traveled 10 cm up the plate, the plate was removed from the chamber, dried in air, and sprayed with 5% stannous chloride solution. The plate was then heated for 10 min at 100°C. It was cooled and sprayed with freshly prepared 5% sodium nitrite solution in 10% (v/v) acetic acid, followed by 1-naphthylamine reagent. Ethyl parathion,
Table 1. RF values for 3 organophosphorus insecticides containing a nitrophenyl group

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl parathion (Ekatox, Follidon)</td>
<td>0.60</td>
</tr>
<tr>
<td>Methyl parathion (Metacide-50)</td>
<td>0.35</td>
</tr>
<tr>
<td>Fenitrothion (Follithion, Sumithion)</td>
<td>0.50</td>
</tr>
</tbody>
</table>

* Solvent system, hexano-acetone (9 + 1).

methyl parathion, and fenitrothion gave dark pink-orange spots. The RF values are listed in Table 1.

Results and Discussion

Stannous chloride in 50% HCl (v/v) reduces ethyl parathion, methyl parathion, and fenitrothion to their respective amino derivatives (12) on heating at 100°C. The amino derivative so formed is further diazotized by sodium nitrite in acetic acid and coupled with 1-naphthylamine to give a dark pink-orange spot. The pink-orange spot remains stable for a couple of days. Other organophosphorus insecticides (malathion, dimethoate, fenithion, phorate, disulfoton, oxydemeton methyl, quinalphos, phosphamidon, dichlorvos, and trichlorphon) do not give a colored spot. Moreover, organochlorine and carbamate insecticides and various constituents of visceral extracts (peptides, proteins, etc.) do not interfere. The limit of detection of the reagent is about 1 μg.

This reagent also gives a bluish to violet spot with p-nitrophenol (13; RF value, 0.30), a metabolite of ethyl parathion and methyl parathion and with 3-methyl-4-nitrophenol (RF value, 0.25), a metabolite of fenitrothion that is formed in living organisms through biotransformation and also found in commercial formulations of these insecticides as a degradation product. The limit of detection of p-nitrophenol is about 1 μg. Thus, with the help of this reagent, an unchanged insecticide and its active metabolite can be detected and distinguished simultaneously from others present in biological material. Other organic compounds containing aromatic nitro groups and aromatic amino groups may interfere, but such substances do not occur in blanks from untreated individuals and hence do not interfere.

The reagent described here is sensitive and selective for organophosphorus insecticides containing a nitrophenyl group and their active metabolite p-nitrophenol and can be used routinely for the detection and semiquantitative determination of these insecticides in biological materials in forensic toxicology.

Acknowledgment

The authors are grateful to Prof. D. B. Ingle, Head, Department of Chemistry, Marathwada University, Aurangabad, and to the Director, Forensic Science Laboratories, State of Maharashtra, Bombay, for valuable advice and encouragement.

References

Thin-layer Chromatographic Spray Reagent for the Screening of Biological Materials for the Presence of Carbaryl

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Ray reagent for the detection of the carbamate pesticide carbaryl was developed, consisting of 1% ammonium cerium(IV) nitrate in 20% v/v hydrochloric acid. The reagent reacts with the hydrolysis product of carbaryl, phthol, and forms a violet complex. Other carbamate pesticides do not give similar coloured spots. Moreover, naphthoresorcinol, organochlorine and pyrethroid pesticides and constituents of visceral extracts (amino acids, peptides, proteins, etc.) do not interfere. The sensitivity of the reagent is about 0.1 µg.

**Words:** Thin-layer chromatography; ammonium cerium(IV) nitrate; carbaryl; insecticide; biological material

Sodium nitrite solution, 1%. A 1 g amount of sodium nitrite was dissolved in 100 ml of distilled water.

Sodium hydroxide solution, 10%. A 10 g amount of sodium hydroxide was dissolved in 100 ml of distilled water.

**Procedure**

A standard glass TLC plate was coated with a slurry of silica gel G (ACME) in water (1 + 2 m/m), to a thickness of 0.25 mm and the plate was activated at 110 °C for about 1 h. An amount of 1 µl of carbaryl in ethanol (1 mg ml⁻¹) was spotted on the plate, which was then developed in a previously saturated TLC chamber using hexane–acetone (4 + 1) as solvent up to a height of 10 cm.

The plate was removed from the chamber, dried in air and sprayed with 10% sodium hydroxide solution, followed by freshly prepared 1% ammonium cerium(IV) nitrate reagent. A violet spot was observed immediately on the TLC plate at Rᵢ = 0.45. On spraying 10% sodium nitrite solution on the same plate, the intensity of the colour increased. The hydrolysis product of carbaryl, 1-naphthol, gives a similar colour reaction with the reagent at Rᵢ = 0.54 without previous hydrolysis with sodium hydroxide. It was observed that commercial carbaryl in a formulation gave two spots with Rᵢ values of 0.45 and 0.54, demonstrating that the commercial formulation sometimes contains 1-naphthol.

**Extraction of Carbaryl Insecticide From Biological Material**

Portions of about 100 g each of various types of visceral tissue (stomach, intestine, liver, spleen and kidney) containing carbaryl insecticide were individually minced in aqueous solution. Each sample was extracted in a separating funnel with 150 ml of diethyl ether, shaking the funnel for 2–3 min. The ether extract was transferred into an evaporating dish. The aqueous phase was re-extracted with 50 ml of diethyl ether (2–3 times). The extracts were combined and the solvent was evaporated at room temperature. The residue was dissolved in 1–2 ml of ethanol. A known volume (10 µl) of the solution was spotted on an activated TLC plate together with the standard solution of carbaryl insecticides. The plate was then developed as described under Procedure and sprayed with 10% sodium hydroxide solution followed by 1% ammonium cerium(IV) nitrate reagent and sodium nitrite solution.

**Experimental**

Reagents

Reagents were of analytical-reagent grade. Distilled water was used throughout.

Ammonium cerium(IV) nitrate reagent, 1%. A 1 g amount of ammonium cerium(IV) nitrate was dissolved in 100 ml of 20% hydrochloric acid.

Sodium nitrite solution, 1%. A 1 g amount of sodium nitrite was dissolved in 100 ml of distilled water.

Sodium hydroxide solution, 10%. A 10 g amount of sodium hydroxide was dissolved in 100 ml of distilled water.

**Results and Discussion**

A 1 mg amount of carbaryl was added to 100 g of minced visceral tissue, mixed well and kept for 1 d. The insecticide was then extracted with diethyl ether, the solvent evaporated at room temperature and the residue dissolved in 1 ml of ethanol. A 10 µl volume of this solution was spotted on a preactivated TLC plate together with 10 µl each of standard technical carbaryl solutions containing 80, 90, 100 and 110 µg
of carbaryl per 100 ml of ethanol. The plate was then developed as described under Procedure and sprayed with 10% sodium hydroxide solution followed by 1% ammonium cerium(IV) nitrate reagent and 10% sodium nitrite solution. The intensity of the coloured spot for the visceral extract was compared with those obtained for the known standards and was found to correspond to the spot representing a concentration of 100 mg per 100 ml (average of three experiments). Hence the recoveries appeared to be better than 90%.

This reagent does not react with propoxur and carbofuran but does react with their hydrolysis products, giving reddish orange spots (sensitivity approximately 10 μg). Moreover, the following gave no reaction with this reagent and hence did not interfere: organophosphorus insecticides such as malathion, parathion, dimethoate, fenthion, fenitrothion, monocrotophos, methyl demeton, quinalphos, phosalone, ekatin, phorate, phosphamidon, dichlorvos and trichlorfon; organochlorine insecticides such as endrin, DDT, γ-HCH; and endosulfan and pyrethroid insecticides such as fenvalerate, cypermethrin and deltamethrin. The sensitivity of the reagent is approximately 0.1 μg per spot, observed after development.

On alkaline hydrolysis, carbaryl gives 1-naphthol, which then reacts with ammonium cerium(IV) nitrate in acidic media to give the violet complex as shown in Fig. 1.

The authors thank Professor D. B. Ingle, Head of the Department of Chemistry, Marathwada University, Aurangabad, and the Director, Forensic Science Laboratories, State of Maharashtra, Bombay, for their keen interest and valuable guidance in this work.

Fig. 1 Proposed reaction for formation of coloured complex

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A NEW SPRAY REAGENT FOR SELECTIVE DETECTION OF DICHLORVOS BY THIN-LAYER CHROMATOGRAPHY

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(Received 12 August 1993. Accepted 14 September 1993)

Summary—The misuse of dichlorvos (DDVP), an organophosphorus insecticide, results in many instances of poisoning. This paper describes a new spray reagent for selective detection of dichlorvos in biological materials by thin-layer chromatography. Dichlorvos in presence of moisture breaks down to dichloroacetaldehyde which in turn reacts with phenylhydrazine hydrochloride to give a yellowish red colour. In acidic media the colour is intensified and consequently the sensitivity of detection increases. The reagent is selective for dichlorvos, other organophosphorus insecticides failed to give a coloured spot. Moreover organochlorine, carbamate and synthetic pyrethroid insecticides or even constituents of visceral extracts (amino acids, peptides, proteins etc.) do not interfere. The limit of detection is ca 10 μg.

ichlorvos (DDVP), an organophosphorus insecticide, is a systemic insecticide and acaricide, used to control sucking, chewing and boring sects and spider mites on a very wide range of ops. Unfortunately, its ready access has resulted in its increased misuse in homicidal and acidal poisoning. Hence the need has therefore arisen for a rapid and reliable method for the detection and determination of dichlorvos in biological materials.

Very few reagents have been described in the literature for the detection of dichlorvos by in-layer chromatography. Most common spray reagents are bromine-fluorescein-silver nitrate,1 zinc-chloride-diphenylamine,2,3 alcoholic tolidine or o-dianisidine and irradiation with V light,4 and 3,3',5,5'-tetramethylbenzidine,5 or detection of chlorine containing organophosphorus insecticides and organochloro insecticides in general and ethanolic resorcinol containing 5% of sodium hydroxide for dichlorvos and trichlorfon.

In this paper we proposed a new spray reagent viz. 1% w/v aqueous phenylhydrazine hydrochloride followed by 10% v/v hydrochloric acid for detection of dichlorvos, by TLC. The reagent is selective for dichlorvos, other organophosphorus insecticides failed to give a coloured spot. Moreover organochlorine, synthetic pyrethroids and carbamate insecticides, and coextractives from visceral material (amino acids, peptides, proteins, etc.) do not interfere. The limit of detection of the reagent is ca 10 μg per spot, after development.

EXPERIMENTAL

Reagents

All reagents used were of analytical reagent grade. Distilled water was used throughout.

Silica gel G, particle size 15 μm with 13% CaSO₄ binder (Merck, Darmstadt, FRG) was employed to prepared TLC plates.

A dichlorvos stock solution (1 mg/ml) was prepared by dissolving 13.15 mg of, 76% technical grade dichlorvos (Hindustan Ciba-geigy Bombay, India) in 10 ml of ethanol.

Aqueous phenylhydrazine hydrochloride reagent (1% w/v) was prepared by dissolving 1 g of phenylhydrazine hydrochloride (Qualigen, Bombay, India) in 100 ml distilled water and filter. Hydrochloric acid (10% v/v) was prepared by diluting 10 ml of con. HCl (32%) to 100 ml with distilled water.

Extraction of dichlorvos from biological material

A portion of ca 100 g each of various types of visceral tissue (stomach, intestine, liver, spleen and kidney) containing dichlorvos was
individually minced in an aqueous solution. Each sample was extracted in a separating funnel with 150 ml of diethyl ether, shaking the funnel for 2–3 min. The ether extract was transferred into an evaporating dish. The aqueous phase was re-extracted with 50 ml of diethyl ether (2–3 times). The extract was combined and the solvent was evaporated at room temperature. The residue was dissolved in 1–2 ml ethanol. A known volume (10 μl) of the solution was dropped onto an activated TLC plate together with the standard solution of dichlorvos insecticide. The plate was then developed as described under TLC procedure and sprayed with 1% w/v aqueous phenylhydrazine hydrochloride reagent followed by 10% v/v hydrochloric acid solution.

**TLC procedure**

A standard glass TLC plate was coated with a slurry of silica gel G in water (1 + 2) to a thickness of 0.25 mm. The plate was activated at 110°C for about 1 h. Volume of 10 μl of standard solution of dichlorvos in ethanol (1 mg/ml) was dropped on the plate, which was then developed in a previously saturated TLC chamber using n-hexane:ethyl acetate:methanol (14 + 3 + 3) as the solvent. After the solvent had eluted a distance of 10 cm up the plate, the latter was removed from the chamber dried in air and sprayed with 1% w/v phenylhydrazin hydrochloride reagent, after 5 min yellowish red spot appeared. On spraying with 10% v/v hydrochloric acid, an intense red colored spot develops at \( R_F = 0.5 \).

\[
\begin{align*}
\text{CH}_3\text{O} & \quad \text{O} & \quad \text{C} & \quad \text{C} & \quad \text{Cl} & \quad \text{H}_2\text{O} & \quad \text{CH}_3\text{O} \\
\text{CH}_3 & \quad \text{O} & \quad \text{Cl} & \quad \text{Cl} & \quad \text{O} & \quad \text{Cl} & \quad \text{Cl} \\
\text{(I) Dichlorvos} & \quad \text{(II) Dimethyl-phosphoric acid} & \quad \text{(III) Dichloroacetaldehyde} \\
\end{align*}
\]

\[
\begin{align*}
\text{Cl} & \quad \text{H} & \quad \text{H} & \quad \text{H} & \quad \text{N} & \quad \text{N} & \quad \text{NH} & \quad \text{Cl} & \quad \text{Cl} & \quad \text{H}_2\text{O} & \quad \text{NH} & \quad \text{Cl} & \quad \text{Cl} & \quad \text{N} & \quad \text{N} & \quad \text{NH} & \quad \text{H} & \quad \text{H} & \quad \text{Cl} \\
\text{(III) Dichloroacetaldehyde} & \quad \text{(IV) Phenylhydrazine} & \quad \text{(V) Dichloroacetaldehyde-phenylhydrazine (coloured-species)} \\
\end{align*}
\]

**RESULTS AND DISCUSSION**

**Recovery experiment**

A 100 mg amount of dichlorvos was added to 100 g of minced visceral tissue, mixed well and kept for a day. The insecticide was then extracted with diethyl ether as described under Extraction of dichlorvos and the solvent was evaporated at room temperature. The residue was then dissolved in 100 ml of ethanol. A 10 μl volume of this solution was dropped onto an activated thin-layer plate together with 10 μl each of standard technical dichlorvos solutions containing known concentrations of 80, 90, 100 and 110 mg of dichlorvos per 100 ml of ethanol. The plate was then developed as described under the previous section and sprayed with 1% w/v phenylhydrazine hydrochloride reagent, followed by 10% v/v hydrochloric acid solution. The intensity of the yellowish red spot developed for the spiked visceral extract were compared with those obtained for the known standards and was found to correspond to the spot representing a concentration of 90 mg per 100 ml (average of three experiments). Hence the recovery was ca 90%

This reagent is selective for dichlorvos. Other organophosphorus insecticides, such as phosphamidon, monocrotophos, malathion, parathion, dimethoate, quinalphos, phorate, fenthion, fenitrothion, and phosalone; organochlorine insecticides such as endrin, aldrin, dieldrin, endosulfan, DDT and BHC; carbamate insecticides such as propoxur, carbaryl, carbosulfan, and synthetic pyrethroid insecticides, such as fenvalerate, cypermethrin and

![Fig. 1. Proposed reaction for formation of coloured species.](image-url)
Itamethrin do not give coloured spot. Moreover constituents of viscera (amino acid, peptides, proteins, etc.) which are generally -extracted with the insecticides, do not interfere. The sensitivity of the reagent is ca 10 μg per spot observed after development.

Dichlorvos in the presence of moisture, breaks down to acidic products, dimethylphosphoric acid and dichloroacetaldehyde, which further catalyse the decomposition. The dichloroacetaldehyde thus formed further reacts with phenylhydrazine to give yellowish red coloured dichloroacetaldehyde phenylhydrazine complex, shown in the following scheme (Fig. 1). The colour of the spot is stable for a couple of days.

The reagent described here is sensitive and specific for dichlorvos and hence can be used routinely for the detection and determination of carbayl in biological and non-biological materials in forensic toxicology.

Acknowledgements—The authors are grateful to Prof. D. B. Ingle, Head Department of Chemistry, Marathwada University, Aurangabad and to S. O. Baigane, Director, Forensic Science Laboratories, State of Maharashtra, Bombay, for their valuable advice and encouragement in this work.

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Short Communication

Thin-layer chromatographic detection of carbaryl using phenylhydrazine hydrochloride

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Abstract

A new chromogenic spray reagent for the detection of the commonly misused carbamate insecticide carbaryl is described. Carbaryl, on alkaline hydrolysis, yields 1-naphthol, which in turn reacts with phenylhydrazine hydrochloride to give a red complex. This reagent is selective for carbaryl. There is no interference from other carbamate insecticides or from organophosphorus, organochlorine and pyrethroid insecticides or from constituents of visceral extracts (amino acids, peptides, proteins, etc.). The limit of detection of the reagent is ca. 0.1 μg per spot (i.e. ca. 350 ng/cm²) observed after development.

Introduction

Carbaryl, 1-naphthyl N-methyl carbamate, is a broad contact insecticide with occasional systemic activity. It is used for pest control in India and any tropical countries. Its use is continually crossing, and this is reflected in the increasing number of criminal cases referred to forensic science laboratories concerning the misuse of carbamates. Hence, its selective characterization is necessary. A number of reagents have been used for its detection by thin-layer chromatography (TLC), namely diazophenol (after alkaline hydrolysis) [1], alkaline fast blue B [2] and ollen's reagent [3]. However, these reagents are normally used for phenolic compounds or cannabinoids, and are susceptible to biological impurities such as amino acids, proteins and peptides and are not specific. Although a copper (II) chloride followed by ammonium metavanadate reagent [4] is reported to be specific for carbaryl, it has a low sensitivity of detection.

In this paper we report the use of 1% phenylhydrazine hydrochloride in an alkaline medium for the detection of carbaryl by TLC, yielding an intense red colour.

Experimental

Reagents

All reagents were of analytical-reagent grade. Distilled water was used throughout.

Alkaline phenylhydrazine hydrochloride reagent. Equal volumes of 1% (w/v) aqueous phenylhydrazine hydrochloride solution and 10% (w/v) aqueous sodium hydroxide solution are mixed together just before use.

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Extraction of carbaryl from biological materials

Portions of ca. 50 g each of various types of visceral tissue (stomach, intestine, liver, spleen and kidney) containing carbaryl were individually minced in 50 ml of aqueous solution. The insecticide was then extracted with 200 ml of diethyl ether and the solvent was evaporated at room temperature. The residue was dissolves in 1–2 ml of ethanol. A known volume (10 μl) of the solution was spotted on an activated TLC plate together with the standard solution of insecticide. The plate was then developed as described in the Procedure section and sprayed with alkaline phenylhydrazine hydrochloride reagent.

Procedure

A standard glass TLC plate was coated with a slurry of silica gel G in water (1:2) to a thickness of 0.25 mm. The plate was activated at 110°C for about 1 h. A 10-μl volume of a standard solution of carbaryl in ethanol (1 mg/ml) was spotted on the plate, which was then developed in a previously saturated TLC chamber using n-hexane–acetone (4:1) as the solvent up to a height of 10 cm. The plate was removed, dried in air and sprayed with alkaline phenylhydrazine hydrochloride reagent. An intense red spot was observed immediately on the TLC plate at an R_F value of 0.45.

RESULTS AND DISCUSSION

Recovery experiment

A 1-mg amount of carbaryl was added to 50 g of minced visceral tissue, mixed well and kept for a day. The insecticide was then extracted with diethyl ether, the solvent was evaporated at room temperature and the residue was dissolved in 1 ml of ethanol. A 10-μl volume of this solution was spotted on an activated thin-layer plate together with 10 μl each of standard technical carbaryl solutions containing known concentrations of 9, 9.5 and 10 mg per 10 ml in ethanol. The plate was then developed as described in the Procedure section and sprayed with alkaline phenylhydrazine hydrochloride reagent. The intensity of the red spots developed from the visceral extracts was compared with those of the known standards and found to agree with the spot resulting from a carbaryl concentration of 10 mg/10 ml (average of three experiments). Hence the recovery was ca. 100%.

This reagent is selective for carbaryl. Other carbamate insecticides, such as baygon, carbosulfan and Zineb, organophosphorus insecticides, such as malathion, parathion, dimethoate, quinalphos, phorate, fenitrothion and monocrotophos, organochlorine insecticides, such as endrin, aldrin, dieldrin, endosulfan, DDT and benzene hexachloride, and pyrethroid insecticides, such as fenvalate, cypermethrin and deltamethrin, do not give a coloured spot. Moreover, constituents of viscera (amino acids, peptides, proteins, etc.), which are generally coextracted with the insecticides, do not interfere. The sensitivity of the reagent is ca. 0.1 μg per spot (i.e. ca. 353 ng/cm^2) observed after development.

On alkaline hydrolysis carbaryl yields 1-naphthol [5,6], which then reacts with phenylhydrazine hydrochloride to give red complex III, as shown in Fig. 1. Technical-grade carbaryl and 1-naphthol give one spot at R_F 0.45 and 0.54, respectively, whereas carbaryl in formulation and extracts of biological materials from patients with carbaryl poisoning give two spots with R_F values of 0.45 and 0.54, demonstrating that they contain the hydrolysis product.

![Fig. 1. Proposed reaction for formation of coloured species.](image-url)
naphthol. The colour of the spots is stable for a few days.

The reagent described here is very sensitive and specific for carbaryl and hence can be used reliably for the detection and determination of carbaryl and its breakdown product, 1-naphthol, in biological and non-biological materials in forensic toxicology.

ACKNOWLEDGEMENTS

The authors are grateful to Professor D.B. Patil, Head, Department of Chemistry, Marathwada University, Aurangabad, and to the Directorate of Forensic Science Laboratories, State of Maharashtra, Bombay, for their valuable advice and encouragement in this work.

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Thin Layer Chromatographic Detection of Certain Benzodiazepines

Vithal B. Patil * and Muridhar S. Shingare

Key Words:
Benzodiazepines
Chromogenic reagent
Hydrolasys
Diazotization
1-Naphthylamine

1 Introduction

Benzodiazepines are most commonly employed as psychotherapeutic, tranquilizing, sedative, and hypnotic drugs; they are widely used in the treatment of nervous diseases, such as epileptic convulsions, insomnia, and anxiety. Because, owing to their ready availability, they are frequently encountered in forensic casework samples involving drug overdoses, a rapid method is needed to characterize the compounds in biological materials.

Methods reported for the detection and determination of 1,4-benzodiazepine derivatives include spectrophotometry [1], gas chromatography [2-4], polarography [5,6], high performance liquid chromatography [7-9], and radioimmunoassay [10]. Thin layer chromatography has also been used to advantage; Meelos et al. [11] have identified benzodiazepines by TLC on fluorescent silica gel GF254.

Reagents are available for detection of benzodiazepines by TLC include:

- Dragendorff's reagent [12];
- hydrolysis by heating with hydrochloric acid for 30 min and then diazotization and coupling with 1-naphthol [13];
- heating with 2,5-dimethoxytetrahydrofuran followed by reaction with p-dimethylaminobenzaldehyde [14]; and
- exposure to nitrogen dioxide fumes, activation, and then spraying with Bratton-Marshall reagent (0.1 % N-(1-naphthyl)ethylenediamine in ethanol) [15,16].

In this paper we report the hydrolysis of certain benzodiazepines, e.g. nitrazepam and oxazepam, to their 2-aminobenzophenones which are then reduced by heating at 110 °C for 10 min with stannous chloride in 1:1 hydrochloric acid [13]. Addition of 10 % ammonium chloride solution converts the amine salt thus formed to the free base which is then further diazotized and coupled with Griess reagent (0.1 % 1-naphthylamine) giving intense pink spots. The various constituents of visceral extracts (amino acids, peptides, proteins, etc.) do not interfere.

2 Experimental

2.1 Reagents

All reagents were of analytical reagent grade; distilled water was used throughout.

1 % Stannous chloride solution was prepared by dissolving stannous chloride (1 g) in hydrochloric acid (50 % v/v; 100 ml). 10 % Ammonium chloride solution was prepared by dissolving ammonium chloride (10 g) in distilled water and diluting to 100 ml. 1 % Sodium nitrite solution was prepared by dissolving sodium nitrite (1 g) in hydrochloric acid (20 % v/v; 100 ml). Griess reagent (0.1 %) was prepared by dissolving 1-naphthylamine (0.1 g) in glacial acetic acid (10 ml) and diluting to 100 ml with distilled water.

2.2 Extraction of Benzodiazepines from Biological Material

Samples (ca 50 g) of various types of visceral tissue (stomach, intestine, liver, spleen, and kidney), and/or blood/urine (10 ml) in cases of benzodiazepine drug overdoses, were individually minced in aqueous solution. The drug being sought was extracted with diethyl ether under alkaline conditions and the solvent evaporated at room temperature. The residue was dissolved in ethanol (1-2 ml).

2.3 Chromatography

Standard glass TLC plates were coated with 0.25 mm layers of a slurry of silica gel G and water (1:2). The plates were activated by heating at 110 °C for ca 1 h.

Aliquots (10 μl) of the ethanolic solution from sample preparation (Section 2.2) and a standard solution of nitrazepam and oxazepam were spotted on to an activated TLC plate which was then developed with hexane – acetone (6 + 4, v/v) in a previously saturated TLC chamber. The development distance was 10 cm. The plate was removed from the chamber, dried in air, sprayed with stannous chloride rea-
gent, and then heated at 100 °C for 10 min. After cooling, the plate was sprayed with 10 % ammonium chloride solution and then, after 5 min, with sodium nitrite reagent followed by 0.1 % Griess reagent. Pink spots were observed immediately and became more intense after 10-15 min.

Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>Spot color with Griess reagent</th>
<th>Dragendorff’s reagent</th>
<th>hRf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrazepam</td>
<td>Pink</td>
<td>–</td>
<td>74</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>–</td>
<td>Orange</td>
<td>55</td>
</tr>
<tr>
<td>Diazepam</td>
<td>–</td>
<td>Orange</td>
<td>85</td>
</tr>
<tr>
<td>Medazepam</td>
<td>–</td>
<td>Orange</td>
<td>95</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>–</td>
<td>Orange</td>
<td>61</td>
</tr>
</tbody>
</table>

3 Results and Discussion

The Rf values of nitrazepam and oxazepam are given in Table 1.

Nitrazepam and oxazepam are 1,4-benzodiazepine derivatives which on hydrolysis and reduction with acidic stannous chloride solution give the corresponding 2-aminobenzophenone. The amine salts thus formed are converted to the free base by addition of ammonium chloride and then further diazotized and coupled with 1-naphthylamine (Griess reagent), giving intense pink spots. The color of the spots remains stable for two days. In addition to the above hydrolysis product, nitrazepam also contains a nitro group in its molecule which is also reduced to an amino group; this gives the compound enhanced reactivity toward the azo dye reagent. The limits of detection of the reagent are ca 0.1 and 1 μg for nitrazepam and oxazepam, respectively.

Benzodiazepines with methyl substitution in the 1-position, i.e. diazepam, medazepam etc., do not react with the 1-naphthylamine reagent, but may be visualized by spraying (the same plate) with modified Dragendorff’s reagent. The reagents used previously do not interfere with Dragendorff’s reagent. Diazepam, medazepam, and chlordiazepoxide give orange spots and the pink spots of nitrazepam and oxazepam turn dark violet. The Rf values of diazepam, medazepam, and chlordiazepoxide are also given in Table 1.

Amino compounds such as dapsone and sulphonamides, also of forensic interest, react with this reagent, without prior reduction, to form azo dyes; the reagent also reacts with nitrophenyl compounds previously reduced with acetic stannous chloride reagent. The limit of detection of the reagent for dapsone and sulphonamide drugs is ca 0.1 μg. The reagent is both sensitive and extremely simple to use and hence can be used routinely in forensic toxicology for the detection and semi-quantitative determination of certain benzodiazepines and their benzophenone metabolites in biological materials.

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References


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