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Note

Thin layer chromatography of apomorphine and its analogs

PAUL W. ERHARDT, ROBERT V. SMITH, and TERRY T. SAYTHER

Drug Dynamics Institute, College of Pharmacy, The University of Texas at Austin, Austin, Texas 78712 (U.S.A.)

and

JEFFREY E. KEISER

Department of Chemistry, Coe College, Cedar Rapids, Iowa 52402 (U.S.A.)

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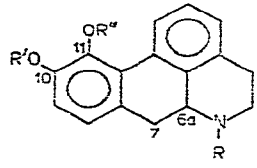
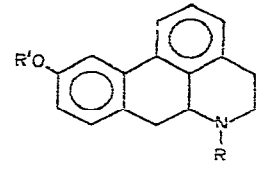
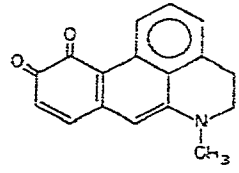
As part of systematic studies concerning the mammalian metabolism of aporphine alkaloids[1–3], recent investigations in these laboratories have focused on the metabolic fate of apomorphine analogs which are potential anti-parkinsonian prodrugs[4–7].



(I) Apomorphine. R = CH_3; R′ = R″ = H
(II) Apocodeine: R = R′ = CH_3, R″ = H.
(III) Isoapocodeine: R = R″ = CH_3, R′ = H.
(IV) Apomorphine dimethyl ether: R = R′ = R″ = CH_3.
(V) 10,11-Dihydroxy-6-*n*-propylnoraporphine R = $(CH_2)_2CH_3$, R′ = R″ = H.



(VI) 10-Hydroxyaporphine: R = CH_3; R′ = H.
(VII) 10-Methoxyaporphine: R = R′ = CH_3.
(VIII) 10-Hydroxy-6-*n*-propylnoraporphine: R = $(CH_2)_2CH_3$; R′ = H.



(IX) Apomorphine orthoquinone.

The instability of compound I due to air oxidation of the catechol moiety[8], and the sensitivity toward oxidation of the C_{6a} and C_7 positions of aporphines[9], are well known. Accordingly, preliminary investigation indicated that significant decomposition of these materials occurs during typical microsomal metabolism studies, *e.g.*, during both microsomal incubation and drug metabolite isolation procedures. Because of this labile nature, methods were developed to prevent air oxidation of these materials during each of the steps encountered in metabolic studies. Decomposition in aqueous media or under microsomal incubation conditions can be effectively prevented by use of antioxidants (sodium bisulfite or dithiothreitol) while decomposition during isolation procedures can be prevented by rigorous nitrogenation of the organic solvents used to extract and concentrate samples prior to analysis. Employing these techniques, thin-layer chromatography (TLC) of I–VIII yields single, characteristic spots for each of the compounds. TLC solvent systems have been developed to separate the metabolic substrates II–VIII from I and/or other probable metabolites. These systems and structural characterization of the major decomposition product of I are described in this report

MATERIALS AND METHODS

Materials

Apomorphine hydrochloride hemihydrate was purchased from Penick. Apocodeine (II) was prepared as described previously[3]. Isoapocodeine (III) and 10,11-dimethoxyaporphine (IV) were synthesized according to the procedure of Cannon *et al.*[2]. Compounds V and VIII were obtained through the scheme of Neumeyer *et al.*[10] while VI and VII were made using procedures described by Borgman[11]. Compound IX was prepared according to the method of Linde and Ragab[8]. When the melting point of IX was determined in the usual way, it appeared to decompose without melting, consistent with the reports of previous workers. However, IX rapidly melted when placed in a pre-heated oil bath (200°). All solvents and reagents were reagent grade. Sodium bisulfite SO_2 lot analysis was 59.9%.

Standard solutions

1–5-mg quantities of compounds I–VIII as their hydrohalide salts were dissolved in 1 ml of rigorously nitrogenated water. The aqueous media were neutralized with 1 ml 1.0 *M* Tris–HCl buffer (pH 7.2) and extracted with 2 ml nitrogenated ethyl acetate. Ethyl acetate solutions were used immediately to spot thin-layer plates.

Thin-layer chromatography

Silica gel GF_{254} plates, 250 μm thick (Analtech), were scored into 1-cm channels and spotted with 1–5 μl of standard (ethyl acetate) or 0.25–0.50 ml of sample [concentrated microsomal extracts; chloroform–methanol (1:1)] solutions. Spotted plates were developed 10 cm in the following solvent systems: A, benzene–methanol (4:1); B, acetone–methanol (1:1); C, benzene–ethyl acetate–diethylamine (6:3:1); D, chloroform–acetone (8:2). Visualization was done by quenching of plate fluorescence while irradiated with ultraviolet (UV) light (254 nm) and by use of diazotized sulfanilic acid (DSA) and 2,6-dichloroquinone-4-chloroimide (DCQ) spray reagents. DSA spray reagent was generated immediately prior to use by combining equal

volumes of 0.5% aqueous sulfanilic acid hydrochloride (5 g sulfanilic acid and 53 ml conc. HCl in 1 l of water) and 0.5% aqueous sodium nitrite stock solutions. DCQ spray reagent contained 2% DCQ in methanol. After spraying plates with DCQ, a 15-min interval was allowed for color production at room temperature. The plates were then warmed over a hot plate and colors again noted.

Simulated microsomal metabolism studies

2-μmole quantities of I–VIII in 0.5 ml of 0.01 *N* HCl, were added to simulated microsomal metabolic incubation media containing 300 μmoles Tris–HCl buffer (pH 7.4) and 0.5 g liver tissue (Sprague Dawley rat liver 10.000 *g* supernatant[6,7]) in 3 ml water. Media also contained 0.05% sodium bisulfite or 2 μmole dithiothreitol. Mixtures were incubated under air at 37° for 30 min in a Dubnoff metabolic shaker. Protein was removed from these media by acidification with 0.5 ml of 1 *N* HCl followed by either filtration or centrifugation (2000 *g* for 5 min) and retention of the supernatant. Deproteinated aqueous media were neutralized[5] by adding 2 ml of 1.0 *M* Tris–HCl buffer (pH 7.2) and extracted five times with 6-ml portions of rigorously nitrogenated ethyl acetate. Extracts were maintained under nitrogen and after combination, evaporated to dryness under vacuum. The resulting residues were taken up in 1 ml nitrogenated chloroform–methanol (1:1) and these solutions concentrated (to appropriate volumes for TLC application) by gentle warming under rigorous nitrogenation.

Nuclear magnetic resonance (NMR) studies

NMR spectra were obtained in deuterochloroform (Silanor-C, Merck) as saturated solutions with TMS as an internal standard. Proton spectra were recorded with a Varian A-60 NMR spectrometer and ^{13}C spectra were recorded with a Brucker WH-90 FT spectrometer operated at a frequency of 22.615 MHz.

RESULTS AND DISCUSSION

Fig. 1a illustrates a thin-layer chromatogram of I obtained from a simulated microsomal metabolism study where no precautions were taken to prevent air oxidation. The marked decomposition is apparent. The chromatogram in Fig. 1b is obtained from simulated metabolic studies in which rigorously nitrogenated organic solvents, used to extract and concentrate samples prior to their TLC analysis, were employed. The same chromatogram is obtained from aqueous solutions of I which undergo green discoloration as decomposition progresses. The spot pattern is characteristic for I in a variety of solvent systems and may be referred to as the "apomorphine fingerprint". The material with an value of R_F 0.45 is visible under ordinary light as a dark-green spot and is more intense for aqueous solutions of I which have undergone extensive decomposition. The material with R_F 0.35 is initially visible only under UV irradiation. However, after exposure to the air, it gradually turns green and can then be seen in ordinary light. These data suggested that the spot with R_F 0.35 is I and that the spot with R_F 0.45 results from air oxidation of I. Two-dimensional TLC confirmed that the spot with R_F 0.45 could be obtained from the material with R_F 0.35 if 15-min exposure to air were allowed between developments. It was also suspected that the material with R_F 0.45 possessed structure IX.

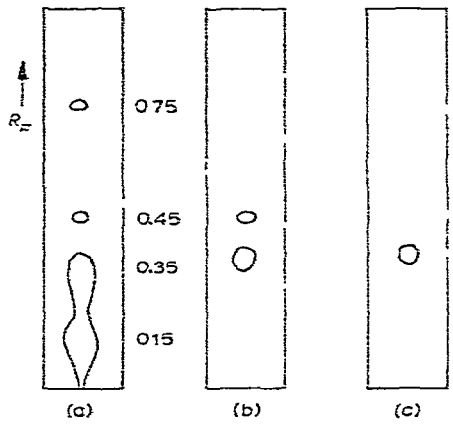


Fig. 1. Thin-layer chromatograms of apomorphine obtained after simulated microsomal metabolism studies. Silica gel G_{254} TLC plates were developed in benzene–methanol (4:1). a, Without antioxidants or nitrogenation: b, with nitrogenation of organic solvents; c, with antioxidants present during microsomal incubation and nitrogenation of organic solvents.

The preparation of IX from I has been reported using either mercuric chloride[8] or potassium dichromate[12]. Repetition of the former procedure produced a dark-green crystalline compound similar to that described by Linde and Ragab[8]. This material possessed R_F values identical to the suspected spot in the apomorphine fingerprint in a variety of developing systems. However, the mass spectrum of this material showed both an expected[8] peak at 263 *m/e* (for $C_{17}H_{13}NO_2$) and an additional unreported peak at 265 *m/e*. The relative abundance of these peaks was found to vary from sample to sample. Although M + 2 peaks are not unusual in the mass spectra of quinones[13,14], it was felt that this descrepancy required further study. Furthermore, the reported[8] lack of a melting point below 350° seemed unusual for a structure such as IX. Since the infrared spectrum and a negative ferric chloride test indicated that phenolic hydroxyl groups were not present, it was suspected that a compound having two additional mass units might then be structure IX where the C_{6a}–C_7 double bond is saturated. NMR studies were conducted to test this possibility. Both proton and ^{13}C spectra were obtained. The proton spectrum was identical to that reported by Linde and Ragab[8] and was appropriate for structure IX. The ^{13}C spectrum is illustrated in Fig. 2, and is also appropriate for structure IX. In this spectrum, the resonances at 183.2 and 176.5 δ may be assigned to C_{11} and C_{10} since these chemical shifts are predicted[15] for α,β-unsaturated ketones and occur at too a low field for aromatic carbons. Other characteristic features are the resonances at 50.4, 28.9 and 40.2 δ which are in the regions expected[16] for C_5, C_4 and N–CH_3 respectively. These studies suggest, then, that the additional peak at 265 *m/e* is an M + 2 artifact which occurs for the

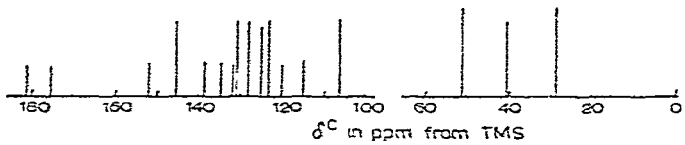


Fig. 2. ^{13}C NMR spectrum of apomorphine orthoquinone obtained in C^2HCl_3.

same material obtained by Linde and Fagab[8] and that the material with an R_F value of 0.45 is indeed IX.

Fig. 1c represents the thin-layer chromatogram of I obtained from simulated microsomal metabolism studies in which antioxidants (either 0.05% sodium bisulfite or equimolar dithiothreitol) were employed during microsomal incubation and rigorous nitrogenation of all organic solvents was employed during subsequent extraction prior to TLC analysis. It is apparent that when metabolic studies are performed in this fashion, even the characteristic spot resulting from air oxidation of I to IX can be eliminated and a single-spot chromatogram of apomorphine can be obtained. Furthermore, there is no evidence of decomposition occurring during the TLC development *per se* as evidenced by single spots on plates developed under conditions described under *Thin-layer chromatography*. To minimize decomposition prior to TLC, plates are rapidly spotted (using solutions prepared with nitrogenated solvents) and immediately developed. Again, two dimensional TLC with 15-min air exposure between developments confirmed that the characteristic apomorphine fingerprint (Fig. 1b) could be obtained from the chromatogram in Fig. 1c.

Since I is one of the more labile compounds in the series I–VIII, the procedures used to prevent its decomposition provide a means for the utilization of TLC methods in metabolism studies of II–VIII as well.

The four solvent systems A–D were designed to accomplish specific goals based upon anticipated results of metabolic studies of I–VIII. Systems A, C, and D separate II, III, IV, VI, and VII from their potential metabolite I. System D separated VIII from V (a potential hydroxylated metabolite of VIII) while systems A, C, and D separated IV from I–III (possible O-demethylated metabolites of IV) and VII from VI (possible O-demethylated metabolite of VII). Based on a previous report[1] it is anticipated that system B will be useful in distinguishing I–VIII from their N-dealkylated metabolites. R_F values for I–VIII appear in Table I. In most cases, these values were maintained when anticipated metabolic mixtures of I–VIII were spotted, *e.g.* a mixture of IV with II and III. However, slightly lower R_F values were obtained for VII and for II when these materials were co-chromatographed with their O-dealkylated analogs (VI and I, respectively). This lowering of the R_F value is thought to result from an associative interaction between the two molecular species involved.

TABLE I

TLC OF APOMORPHINE AND ANALOGS

For composition of solvent systems A–D, see text. R_F values are averages of 4–7 determinations.

Structure	$R_F \times 100$			
	Solvent A	*Solvent B*	*Solvent C*	*Solvent D*
I	35	65	10	10
II	55	60	60	20
III	25	45	40	10
IV	60	65	65	25
V	50	60	40	20
VI	50	60	35	15
VII	60	60	65	25
VIII	50	60	40	30

Ambiguity resulting from this phenomena is not anticipated during metabolic studies since the use of spray reagents dramatically distinguishes between these compounds.

Although visualization by UV irradiation was effective, the spray reagents DSA and DCQ were also found to be useful in characterizing and distinguishing certain of the compounds I–VIII. The behaviors of I–VIII toward DSA and DCQ are summarized in Table II. Interestingly, the reactivity of the phenolic systems present in certain of these aporphines is such that the spray reagents effectively couple without requiring activation by subsequent spray with a basic reagent[17].

TABLE II

BEHAVIOR OF APOMORPHINE AND ANALOGS TOWARD DSA AND DCQ SPRAY REAGENTS

Structure	*Color*		
	DSA	*DCQ**	*DCQ, heat***
I	gray	gray	darker
II	orange	blue	darker
III	pink	none	brown
IV	pink	none	brown
V	brown	gray	darker
VI	brown	none	brown
VII	pink	none	brown
VIII	brown	none	brown

* When DCQ is used after development in solvent system C, entire plates darken and no useful information is obtained.

** After spraying with DCQ, plates were gently warmed over a hot-plate.

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