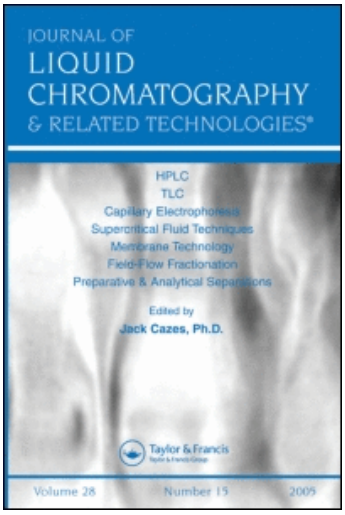

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TLC ANALYSIS OF FORMALDEHYDE PRODUCED BY METABOLIC *N*-DEMETHYLATION

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ABSTRACT

Formaldehyde production was observed during the rat liver microsomal metabolism of (−)-deprenyl. Formaldehyde was reacted to its dimedone adduct. The formaldemethone was identified using thin-layer chromatography, combined with X-ray film autoradiography. Standard samples served for the reliable identification of spots of formaldemethone and those of the other metabolites.

Key Words: Formaldehyde; TLC; Thin layer chromatography; (−)-Deprenyl; Microsomal metabolism

INTRODUCTION

Thin-layer chromatography (TLC) has always been a basic method for monitoring metabolites of exogenous biologically active compounds. A

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quintessential advantage of TLC over the column technique is a choice between either the analysis of several samples at the same time, or two-dimensional separation of a single sample. Also, some of the standards can be used for comparison. The potential applications of certain easy and specific detection procedures give an additional feature of TLC. One of these is that a wide choice of spray reagents may provide reactions that make the detection of the spot specific and sensitive. Combination of thin-layer chromatography and detection of the radiolabelled spot gives a very useful technique to monitor the metabolism of radiolabelled compounds.

Radiolabelling of deprenyl has played a pivotal role in scouting the fate of its metabolites.[1] The metabolites of the radiolabelled (−)-deprenyl are easily detectable by TLC. Using the displacement mode of development,[1–3] the spots of the radiolabelled drugs and their metabolites are highly concentrated.

The analysis of aldehydes, such as formaldehyde, can be performed using chromatography and related separation methods. Especially TLC was used[4–6] for determination of the free formaldehyde. This method generally uses derivatization with dimedone (5,5-dimethyl-1,3-cyclohexanedione), and the formaldemethone (which is the adduct of formaldehyde and dimedone) can be easily determined. Rozylo et al.[6] used TLC for determination of formaldehyde in hard tissues.

High-performance liquid chromatography (HPLC) has also been used for aldehyde determination. Yu[7] dealt with the effect of SSAO (semicarbazide-sensitive amine oxidase) mediated deamination. He determined both formaldehyde and methylglyoxal in rat urine. Derivatization with DNPH (2,4-dinitrophenylhydrazine) resulted in the corresponding dinitrophenylhydrazones. Yu separated the peaks of malondialdehyde, methylglyoxal, and formaldehyde on a column packed with C-18 stationary phase.

Basic books on pharmacology[6–10] discuss the *N*-demethylation as an important reaction belonging to the microsomal, cytochrome P-450 dependent oxidation. The process yields both the demethylated compound and formaldehyde. The characteristics, fate, and significance of the demethylated compounds are generally discussed in detail, but the formation, presence, and importance of the generated formaldehyde is neglected.

Deprenyl metabolism deals with two basic reactions, both of which are cytochrome P-450 dependent oxidations. One oxidation takes place on the nitrogen; the other one is *p*-hydroxylation. The results of oxidation on the nitrogen may be *N*-demethylation, *N*-depropynylation, the combination of *N*-demethylation and *N*-depropynylation, and oxidative deamination. Oxo compounds can be produced via *N*-demethylation (formaldehyde), *N*-depropynylation (propynyl-aldehyde), and oxidative deamination (phenylacetone). This paper concerns the formation of formaldehyde from ^{14}C-labelled deprenyl by the use of microsomal metabolism.

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EXPERIMENTAL

Solvents and chemicals were purchased from commercial sources with the best available purity.

Radiolabelled (−)-Deprenyl

Radiolabelled (−)-deprenyl [(−)-C^{14}-*N*-methyl-*N*-propynyl-phenyl-isopropylamine, 932 MBq/mmol; 3.6 MBq/mg] was prepared from (L)-*N*-propynyl-phenyl-isopropylamine. Radiolabelled (−)-deprenyl was prepared and supplied by the Institute of Isotopes Co., Ltd., Budapest, Hungary.

Thin-Layer Chromatography

TLC silica plates F_{254} on plastic foil (200 × 200 mm, layer thickness 0.2 mm) were purchased from E. Merck (Darmstadt, Germany). The samples were spotted using a 50 μL Hamilton syringe (Bonaduz, Switzerland). The spots were dried with the help of a cold air stream. Separations were performed using the following mobile phases: solvent system No. 1: chloroform–methanol–25% ammonia solution (100 : 10 : 1), and solvent system No. 2: chloroform–triethanolamine (10 : 1). The developments were performed in a glass TLC chamber with a glass lid. After drying, the radiolabelled spots were located and visualized using x-ray film with an exposure time of 4 days (96 h). Quantitation of the radioactivity of each spot was done by scarping the stationary phase at the spot and measuring in a liquid scintillation spectrometer. Detection of the standards was done under UV light of 254 nm.

Rat Liver Microsome

Five grams of fresh liver from untreated rats was homogenized in 20 mL of 0.01 M Na/K phosphate buffer (pH 7.4) by means of an Ultra-Turrax (Janke and Kunkel KA Werke, Staufen, Germany) homogenizer at 10,000 rpm for 2 min. The mixture was centrifuged at 8000 g, at 2°C for 2 min, then the supernatant was immediately used.

Composition of the reaction medium: 2 mL of nucleus-free liver homogenate (supernatant); 1 mL of 25 μmol mL^{-1} NADP in 0.5 mol L^{-1} Na/K phosphate buffer (pH 7.4) + 1 mL of 50 μmol mL^{-1} nicotinamide in 0.5 mol L^{-1} Na/K phosphate buffer (pH 7.4) + 1 mL of 10 μmol mL^{-1} glucose-6-phosphate in 0.5 mol L^{-1} Na/K phosphate buffer (pH 7.4); 0.1 mL of

0.25 μmol mL^{-1} $MgCl_2$; 0.5 mL N-methyl-^{14}C-deprenyl (1 mg mL^{-1} H_2O). Final volume: 5.6 mL.

Incubation of *N*-Methyl-^{14}C-Deprenyl with Liver Microsome

The mixture was incubated for 1 h at 37°C, then filtered (GF/C Whatman filter).

Treatment of Rats

Male Wistar rats (about 200 g, each) were p.o. treated with 5 mg kg^{-1} radiolabelled (−)-deprenyl. The rats were sacrificed after 1 h of the treatment, the body fluids were collected and the organs were dissected in the usual way. Radioactivity was determined after mixing an aliquot of the body fluid with scintillation cocktail (Packard, Groningen, The Netherlands). In the cases of various organs, the tissue was treated with Soluene (Packard, Groningen, The Netherlands) solution at 60°C for 24 h, then added to the scintillation cocktail. The level of radioactivity is given as dpm (desintegration per minute) in Table 2.

The urine sample and the liver homogenate were mixed with a twice-fold excess of 0.2% (w/v) methanolic dimedone, and centrifuged with 8000 g, at 2°C for 2 min; then the supernatant was used for further studies.

Determination of Formaldehyde

To determine the formaldehyde level, the preparation was mixed with a twice-fold excess of 0.2% (w/v) methanolic dimedone. The mixture was homogenized, and centrifuged, and the supernatant was used for the TLC investigation.

RESULTS

TLC separation of the microsomal treatment of radiolabelled ^{14}C-methyl-deprenyl showed the formation of metabolites. Extraction of the metabolites with methanolic dimedone resulted in a solution that was adequate for the direct TLC analysis in solvent system No. 1. The results are presented in Fig. 1. Dark spots represent the radiolabelled metabolites and the parent (−)-deprenyl, which have been identified with the help of standards. At the same time, the degree of darkness on the x-ray film does not indicate the correct amount of radioactivity in

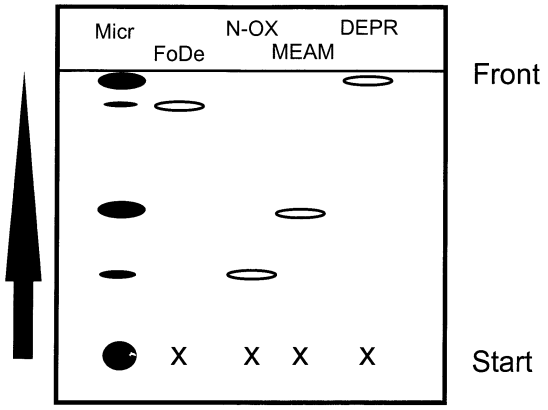

Figure 1. Thin-layer chromatography of rat liver microsomal preparation (Micr) after treatment with methanolic dimedone. The bold spots indicate the dark bands on the x-ray film. For comparison, various standard compounds (FoDe = formaldemethone; *N*-OX = deprenyl *N*-oxide; MEAM = methamphetamine; DEPR = (−)-deprenyl) were subjected to the same TLC. The stationary phase was TLC silica plate F_{254} plastic foil (200 × 200 mm, layer thickness 0.2 mm), and the mobile phase was chloroform-methanol-25% ammonia solution (100 : 10 : 1). The circles indicate darkness under 254 nm UV light.

the spot. The corresponding silica was cut, and the radioactivity was determined. The R_F values and the radioactivity of each spot are given in Table 1.

Two-dimensional TLC also resulted in an adequate separation; however, the spots became more concentrated after displacement development (Fig. 2).

Various body parts of rats were dissected after 1 h of administration of ^{14}C-labelled deprenyl. The radioactivity was determined. Table 2 shows how radioactivity was incorporated in the different parts of the rats.

Table 1. Characteristics of Deprenyl Metabolites Using TLC

Standard Compound	R_F of the Standard	R_F of the Radiolabelled Metabolite	Dpm	%
Unidentified compounds		0.00–0.05	76,000	29.9
Deprenyl *N*-Oxide	0.33	0.35	4,400	1.7
Methamphetamine	0.58	0.57	70,000	27.6
Formaldemethone	0.89	0.89	5,200	2.0
Deprenyl	0.97	0.97	99,000	38.8

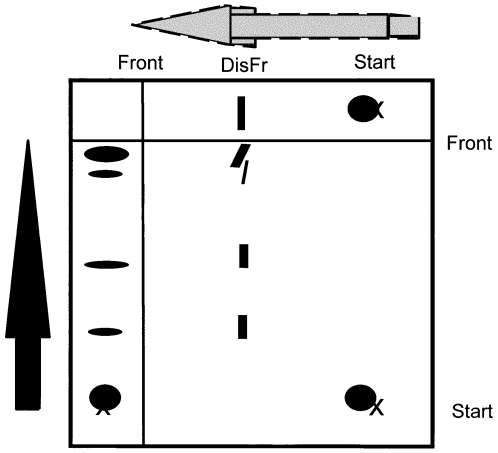

Figure 2. Two-dimensional TLC of rat liver microsomal preparation (Micr) after treatment with methanolic DIMEDONE. The bold spots indicate the dark bands on the x-ray film. The stationary phase was TLC silica plate F_{254} plastic foil (200 × 200 mm, layer thickness 0.2 mm). The first dimensional development was done using chloroform–methanol–25% ammonia solution (100 : 10 : 1). The second dimensional run was performed with chloroform–triethanolamine (10 : 1). The origin, solvent front, and displacer front are indicated with Front, Start, and DisFr, respectively. Spots of the microsomal preparation were separated on the side paths also using unidimensional development.

Table 2. Radioactivity Found in Various Body Compartments of Rats

	Dpm μL^{-1} in Samples of Rat No. 1	Dpm μL^{-1} in Samples of Rat No. 2
Urine	7,550	24,926
Serum	259	218
	Dpm mg^{-1} in Samples of Rat No. 1	Dpm mg^{-1} in Samples of Rat No. 2
Liver	3,863	3,570
Kidney	967	686
Hypophysis	396	333
Parietal cortex	286	232
Frontal cortex	285	237
Hypothalamus	219	184
Brown fat tissue	181	146
Eyes	117	113

The level of radioactivity makes it possible to determine the metabolites from the rat urine. Analysis of radiolabelled deprenyl metabolites will require the use of either clean-up or the use of displacement chromatography.

DISCUSSION

Rat urine and rat liver homogenate contained formaldehyde that was identified using TLC. However, the excess of contaminating compounds and the low level of formaldemethone suggested the use of a clean-up before TLC analysis. The key point was the proper location of labeling, as the monitoring of any non-labeled parts required certain other methods.

TLC of deprenyl metabolites resulted in the indication of its two important metabolites, nordeprenyl (propynylamphetamine) and *p*-hydroxy-methamphetamine. Both of these were neglected earlier, as their levels were much lower than those of the amphetamine and methamphetamine. Moreover, the chromatographic migration of nordeprenyl was very closely related to that of deprenyl, while *p*-hydroxymethamphetamine showed very distinct elution compared to deprenyl. Their discovery was made possible by spacer-displacement thin-layer chromatography, combined with detection of the radiolabelled deprenyl metabolites. Displacement thin-layer chromatography (D-TLC) was performed using silica stationary phase, chloroform carrier, and triethanolamine displacer; it separated all of the deprenyl metabolites. However, D-TLC showed further spots in addition to the usual deprenyl metabolites, which were amphetamine and methamphetamine.[1] One of the additional spots was close to the spot of deprenyl (it was the spot of nordeprenyl); the other one was far from both the spot of deprenyl and also from those of the metabolites, and it was the spot of *p*-hydroxymethamphetamine. The para-hydroxylation essentially changed the chromatographic characteristics of the compound; therefore, *p*-hydroxymethamphetamine did not take part in the displacement train.

The possible presence of formaldehyde in the environment caused difficulties during its quantitative measurement. Our method of choice was radiolabelling the appropriate part of the (−)-deprenyl, and it was the use of *N*-C^{14}-methyl. The occurrence of C^{14}-formaldehyde was, therefore, sure indication of the origin from/by *N*-demethylation. TLC offered a great way for the fast, easy, and inexpensive identification and quantitative determination of C^{14}-formaldehyde using the TLC method published by Tyihák[5,11] and Rozylo.[6]

Our results have indicated the presence of ^{14}C-formaldehyde in the microsomal preparation. The sample of microsomal preparation also gave an adequate sample for two-dimensional separation. The first dimensional development was done using chloroform–methanol–25% ammonia solution (100 : 10 : 1), while the second dimensional run was performed using chloroform-triethanol-

amine (10 : 1). The separation after the first dimensional development was retained, and the displacement process concentrated the spots.

The presence of radiolabelled formaldehyde was also indicated in several different body parts of the rats after 1 h following administration of ^{14}C-labelled deprenyl. However, quantitative evaluation of the metabolic pathway requires further improvements of the TLC separations. Two-dimensional (elution-displacement) TLC seems to be an adequate method to concentrate the spots.

Considering the ratio of radioactivity in the tissue autopsies and body fluid samples, the biological transformation of ^{14}C-deprenyl to ^{14}C-formaldehyde takes place in the liver. Thereby, the high portion of formaldehyde can be present in the liver, kidney, and urine alike. However, essential amounts of radioactivity was also found in certain brain compartments and in the eyes as well.

Formaldehyde is a Janus-faced compound, such as hydrogen peroxide, nitrogen oxide, etc. Being present in high concentration, formaldehyde is a reactive, functional disinfectant, mentioned as environmental pollutant, etc. Its low-level occurrence may play a basic role in the biochemical cycles of human, animal, and plant organisms. It is an essential to ask the question: what is the role of formaldehyde in the treatment of pathological processes of the human and other organisms? The final destiny of the generated formaldehyde may depend on the body part of the human being and/or animal where the formaldehyde is generated. When the formaldehyde was generated in the peripheral part of the body, formaldehyde eliminates with the urine. If formaldehyde is formed in the central nervous system, its transportation outbound is probably hindered by the blood-brain barrier and, therefore, formaldehyde elimination has to take place locally. There are several tentative possibilities for the elimination of formaldehyde, such as the reaction with oxygen free radicals (counterbalancing the oxidative stress); acting as a methylating agent of amino acids, peptides, proteins, nucleic acids, etc.; reacting with another molecule of formaldehyde; etc.

(−)-Methamphetamine does not posses the MAO-B inhibitory action of (−)-deprenyl. However, it has at least two characteristics of (−)-deprenyl. (−)-Methamphetamine acts to increase the dopamine release from the brain corpus striatum, and it also has the *N*-methyl group and is, thereby, a potential formaldehyde precursor.

TLC of radiolabelled deprenyl metabolites served two basic aims. First of all, every radiolabelled spot was detected, including those at the origin and at the solvent front alike. Their quantitative ratio could be determined in an easy and inexpensive way. Two-dimensional development proved the homogeneity of the spots. The second aim was to differentiate the formaldehyde originated from the administered deprenyl from formaldehyde from any other sources, such as the endogenous formaldehyde, formaldehyde from the environment, etc. Monitoring the X-ray film at $R_F = 0.89$ was specific to the formaldehyde originated from the ^{14}C-methyl labeled deprenyl.

TLC has served both of these aims equally. It is for this reason that TLC with the detection of radiolabelled residues has given an easy and useful method for detection and quantitative evaluation of metabolically generated formaldehyde.

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