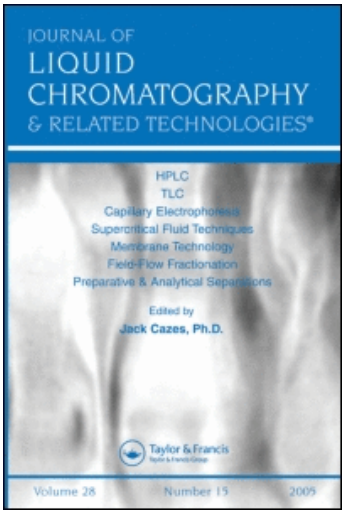

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THE SEPARATION OF N-NITROSO-DI-N-PROPYLAMINE AND ITS B-OXIDIZED CARCINOGENIC METABOLITES BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

N-nitroso-di-n-propylamine (DPN) and three of its B-oxidized derivatives, N-nitroso-bis(2-oxopropyl)amine (BOP), N-nitroso(2-hydroxypropyl)(2-oxopropyl)amine (BHP) were separated by high pressure liquid chromatography (HPLC) with a Waters μ-Porasil column using methylene chloride:2-propanol as the mobile phase. Mass spectrographic data for these four compounds were obtained. Using the newly developed HPLC procedure to determine the conversion of ^{14}C-DPN to its B-oxidized derivatives *in vitro* by hepatic microsomes isolated from the hamster, the formation of ^{14}C-BOP was evidenced in the hepatic microsomes.

INTRODUCTION

DPN is a carcinogenic N-nitrosamine which induces a spectrum of tumors in experimental animals (1-3). Certain B-oxidized derivatives of DPN, such as BOP, HPOP, and BHP, are also potent carcinogens in a variety of animal species, including the Syrian golden hamster (4-11). We report here the development of a new HPLC procedure for the rapid separation of DPN and its B-oxidized derivatives. We also present data on the utilization of this HPLC technique for the separation of B-oxidized derivatives following the *in vitro* metabolism of ^{14}C-DPN by liver microsomal enzymes.

MATERIALS

Chemicals.

N-nitroso-di-n-propylamine-1-^{14}C (^{14}C-DPN, specific activity 2.85 mCi/mmol, >98% pure) was purchased from New England Nuclear (Boston, Mass.). Unlabeled DPN was purchased from Eastmen Organic Chemicals (Rochester, N.Y.). Unlabeled N-nitroso-bis(2-oxopropyl)amine (BOP) and N-nitroso-bis(2-hydroxypropyl)amine (BHP) were purchased from Ash Stevens, Inc. (Detroit, Mich.). Unlabeled N-nitroso(2-hydroxypropyl)(2-oxopropyl)amine (HPOP) was a gift from Dr. P. Pour (Eppley Institute for Cancer Research, Omaha, Nebr.). ^{14}C-DPN, DPN, BOP, BHP and HPOP were used without further purification. Methylene chloride, 2-propanol, and ethyl acetate were obtained from Burdick and Jackson Laboratories, Inc. (Muskegon, Mich.). Prior to use, these solvents were clarified under vacuum through a solvent inert Millipore filter (0.5 um pore size) (Millipore Corporation, Bedford, Mass.) with an all glass filtering apparatus. Reduced nicotinamide adenine dinucleotide phosphate (NADPH), was purchased from Sigma Chemical Co. (St. Louis, Mo.). Insta-Gel was purchased from Packard Instrument Co. (Downers Grove, Ill.). All other chemicals used were of reagent grade quality.

HPLC apparatus.

The HPLC apparatus consisted of a Waters μ-Porasil column (3.9 mm inside diameter x 30 cm)(Waters Associates, Inc., Framingham, Mass.), two Waters Model 6000A high pressure pumps, a Waters Model 660 solvent programmer, a Waters U6K universal injector, a Waters Model 440 ultraviolet (UV) absorbance detector (254 nm), and a Linear Model 300 chart recorder (Linear Instruments Corp., Irvine, Calif.).

METHODS

Mass spectrometry

Mass spectrographic (MS) data on DPN, BOP, BHP, and HPOP

were obtained using a Varian MAT 112S mass spectrometer with fast scan and computerized data acquisition. Samples were introduced using the solid probe with a MS source temperature of 220°C and an ionizing voltage of 72 eV.

In vitro microsomal metabolism

Microsomes from the liver of Syrian golden hamsters (male, 100-120 g) were prepared as described previously (12). The 109,000 x g microsomal pellet was resuspended in an incubation solution containing isotonic KCl (1.15%), 3 mM $MgCl_2$ and 0.05 M TRIS-HCl (pH 7.6). An aliquot of the resuspended hepatic microsomes (1.0 ml) was added to 7.0 ml of the incubation solution, the final mixture containing 1.7mM NADPH and 0.27 mg/ml microsomal protein as determined by the Lowry method (13). The freshly prepared microsomes were preincubated (37°C) for 5 min in a Dubnoff shaker bath prior to the addition of 8.8 uCi ^{14}C-DPN (3088 nmol) in 50 ul isotonic KCl. Following the addition of ^{14}C-DPN, 1 ml aliquots were removed at 0, 5, 10, 15, 20, 25, and 30 min. These aliquots were added to culture tubes containing 2 ml ethyl acetate, mixed, and extracted immediately. The labeled N-nitrosamine compounds were extracted two more times with 2 ml ethyl acetate; the ethyl acetate extractions were then pooled. Prior to injection into the HPLC, the ethyl acetate extract was clarified with Millipore AP organic prefilters and FH fluoropore filters in a Swinny filter holder. To the organic extracts containing ^{14}C-label authentic DPN, BOP, HPOP and BHP standards were added prior to separation by HPLC, using methylene chloride: 2-propanol as the mobile phase at a flow rate of 4 ml/min. Following separation by the HPLC column, 0.4 ml fractions (6 seconds each) were collected in 16 x 25 mm culture tubes, using a LKB 2070 Ultrorac II fraction collector (LKB Instruments, Inc., Bromma, Sweden). The collected samples were mixed with 10 ml of Insta-Gel for determination of radioactivity with a Packard Tri-Carb Model 2650 liquid scintillation spectrometer.

RESULTS

HPLC separation

When only methylene chloride was used as the mobile phase, DPN eluted in 7.9 min (flow rate=3 ml/min) and BOP in 17.7 min (flow rate=8 ml/min); BHP did not elute at all, even when the flow rate of the mobile phase was 8 ml/min. However, when the polarity of the mobile phase was increased by the addition of 2-propanol, the elution was in the following order: DPN, BOP, HPOP, and BHP (Table 1). An increase in elution time appears to be a function of increased polarity of a particular N-nitrosamine using this system. DPN, being the least polar compound, eluted first followed by BOP, HPOP and then BHP. Similar elution position was obtained with thin layer chromatography (TLC) with similar solvent mixtures (unpublished). DPN elution in the void volume was confirmed following co-chromatography with ^{14}C-DPN.

TABLE I

Separation by HPLC[a] of DPN, BOP, HPOP, and BHP with Varying Concentrations of Methylene Chloride (MC) and 2-Propanol (P)

MOBILE PHASE	Elution time (min)				
MC:P	DPN	BOP	HPOP	BHP	BHP IMPURITY
90:10	0.8	1.0	1.1	1.7	-
95:5	0.8	1.0	1.5	3.0	2.6
96:4	0.8	1.0	1.8	3.6	3.0
97:3	0.8	1.1	2.2	4.3	3.8
98:2	0.8	1.2	3.4	6.7	-
99:1	0.8	1.3	7.6	10.4	-

[a]Waters μ-Porasil column; mobile phase flow rate, 4 ml/min; ambient temperature.

Figure 1 shows a typical chromatograph (methylene chloride: 2-propanol, 97:3, flow rate 4 ml/min). While DPN and BOP eluted as single peaks with >99% purity, BHP contained an impurity of lesser polarity that eluted separately and comprised about 2% of this standard. When HPOP was chromatographed alone, two small peaks were noted which, under similar solvent concentrations, eluted in 1.05 and 1.15 min, with each peak representing about 3% of the HPOP standard.

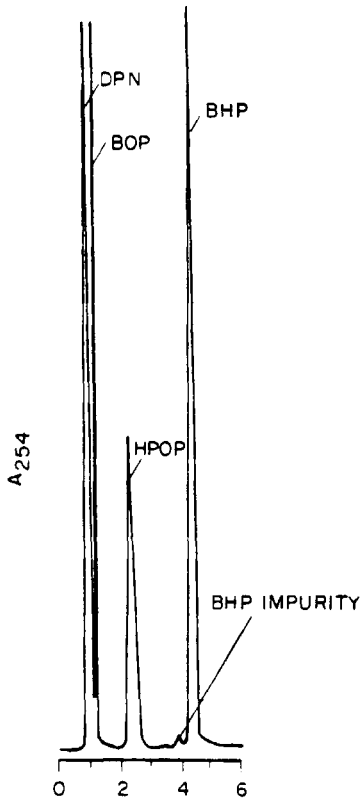

FIGURE 1

Chromatogram of a mixture of DPN, BOP, HPOP and BHP. Conditions: Column, Waters μ-Porasil; mobile phase, methylene chloride: 2-propanol, 97:3; flow rate, 4 ml/min; Δ P = 2000.

Mass spectral data

Table II shows that in the case of all compounds, α-cleavage and the loss of HNO occurred (i.e. $R\text{-}CH{=}\overset{+}{N}{=}CH_2$ or $R\text{-}CH_2\text{-}\overset{+}{N}{\equiv}CH$), and the resultant m/e for the fragments from the parent compounds were: 70.1, DPN; 84.1, BOP; 86.1, BHP and HPOP. The protonated form ($R\text{-}CH_2\text{-}\overset{+}{N}H{=}CH_2$) was also often present (m/e: BOP, 86.1; HPOP and BHP, 88.1). The substituted two carbon fragment(s) resulting from α-cleavage was (were) also observed for BOP (m/e 43.1, base peak), BHP (m/e 45.1, base peak, and 43.1) and HPOP (m/e 43.1 and 45.1). Ions in which only α-cleavage occurred were observed for BOP (m/e 115.1) and for BHP (m/e 117.1). The $CH_2{=}\overset{+}{N}{=}CH_2$ (m/e 42.1) and $CH_2{=}\overset{+}{N}H\text{-}CH_3$ (m/e 44.1) ions were universally present. Cleavage of the C-N bond was also evidenced, accounting for the base peak of DPN (m/e 43.1) as well as a fragment corresponding to the loss of two protons (m/e 41.1). Similar results occurred in the other compounds as well (m/e: 56.9, BOP, HPOP, and BHP; 59.0, BHP). Apparently, in the case of BHP and HPOP, this fragment could cyclize forming a radical with a m/e 58. The $[M\text{-}30]^+$ ion representing the loss of NO was observed for BOP (m/e 128.1), HPOP (m/e 130.2) and BHP (m/e 132.1). Only in BOP was a molecular peak (m/e 158.0) demonstrated. Although no other molecular peaks or $[M\text{-}1]^+$ peaks were observed, in all four compounds the presence of the NO moiety was demonstrated by the thermal energy analyzer (TEA) interfaced to a gas-liquid chromatograph (GLC).

Formation of labeled BOP from ^{14}C-DPN by hepatic microsomes.

Hepatic microsomes isolated from the Syrian golden hamster were incubated for various time intervals with ^{14}C-DPN to determine the formation of any B-oxidized metabolites from this N-nitrosamine. Approximately 79-97% of the radioactivity was recovered corresponded to unmetabolized DPN. However, a portion of the ^{14}C-label extracted from hepatic microsomes co-chromatographed with authentic BOP. Figure 2 shows that the formation of labeled BOP was linear up to 30 min. No label was associated with authentic HPOP and BHP from the same hepatic microsomes.

TABLE II

Mass Spectra Of Tested N-Nitrosamines

DPN		BOP		HPOP		BHP	
MASS	%	MASS	%	MASS	%	MASS	%
43.1	BASE PEAK	43.1	BASE PEAK	42.1	BASE PEAK	45.1	BASE PEAK
56.9	95.7	42.1	54.8	43.1	97.4	42.1	85.0
40.1	82.5	86.1	46.5	41.1	46.7	70.1	62.7
41.1	78.7	58.0	8.7	86.1	42.7	43.1	61.3
71.2	57.3	84.1	8.7	70.1	32.5	88.1	46.6
54.9	55.5	41.1	8.4	45.1	31.4	41.1	46.5
42.1	54.1	85.1	8.4	58.0	22.6	59.0	27.8
70.1	49.9	115.1	7.9	40.1	19.6	86.1	25.8
69.1	41.0	56.9	5.7	39.1	17.8	57.9	16.4
67.1	31.3	44.1	5.?	44.1	13.0	55.9	8.3
83.2	30.7	39.1	5.1	56.9	11.4	44.1	7.9
39.2	29.6	56.0	3.0	88.1	11.0	39.2	7.2
97.1	28.0	40.1	2.4	55.0	8.6	87.1	4.9
55.9	27.2	45.1	1.9	56.0	8.0	71.1	3.9
85.2	26.7	87.1	1.7	130.2	6.9	105.1	3.5
44.0	25.6	128.1	1.6			56.9	3.3
86.1	22.9	68.1	1.4			40.1	2.8
81.2	22.8	38.2	1.2			46.1	2.7
84.1	20.4	69.1	1.1			89.1	2.4
111.2	19.9	37.2	1.1			72.1	2.2
95.1	18.9	59.0	1.0			68.1	1.9
109.2	16.7	158.0	0.3			55.0	1.9
79.1	14.5					132.1	1.8
99.1	14.1					60.0	1.2
82.2	13.7					114.2	1.1
						54.0	1.0
						116.1	1.0
						117.1	0.9
						69.1	0.9
						38.2	0.9
						84.1	0.8
						100.1	0.8

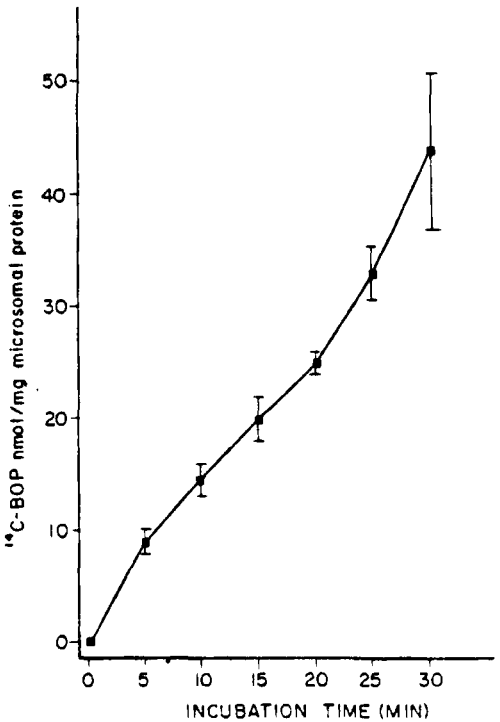

FIGURE 2

The formation of ^{14}C-BOP at various time intervals following the incubation with ^{14}C-DPN of microsomes (109,000 x g pellet) isolated from the hamster liver. Incubation conditions are explained in the text. Values represent X + S.D. (n = 3 experiments of pooled tissue from 3-4 animals).

DISCUSSION

This HPLC technique has several advantages. Separation of DPN, BOP, HPOP and BHP was rapid with clear resolution of the individual compounds. This is a non-destructive technique, superior to TLC and GLC separating procedures (14,15). Unlike certain GLC procedures (14,15), silylation is not needed for separation of the more polar B-oxidized N-nitrosamines (i.e. HPOP and BHP). As reported, this technique can be used in combination with

procedures utilizing radioisotopes. This technique, with slight modifications, if necessary, could also be used to separate other B-oxidized derivatives of DPN such as 2-hydroxypropylpropylnitrosamine, 2-oxopropylpropylnitrosamine, and N-nitrosobis(2-acetoxypropyl)amine. Picogram quantities of unlabeled DPN and its B-oxidized derivatives can be analyzed when this technique is used in conjunction with the TEA; in the present study using a UV detector, sensitivity was ~10 ng for the compounds examined.

The mass spectral data reported here for DPN, BOP, HPOP, and BHP are similar to those reported previously for DPN, BOP, and BHP (16) and HPOP (14). In this study, the most characteristic fragments observed were due to α-cleavage. The m/e 42.1 fragment was also quite evident.

Using this HPLC technique, we have also demonstrated the B-oxidation of ^{14}C-DPN to ^{14}C-BOP by hepatic microsomal enzymes of hamsters; labeled HPOP and BHP were not detected. This technique is expected to be quite useful in the identification of DPN metabolites, such as BOP which is known to be organ specific for the hamster pancreas (10) and HPOP which is believed to be a proximate carcinogenic metabolite (11).

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