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NITROSAMINE FORMATION FROM INTERACTION OF ISOSORBIDE DINITRATE AND HYDROXYZINE HCl, A TERTIARY AMINE

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Abstract—The interaction of two drugs—isosorbide dinitrate (ISDN) and hydroxyzine hydrochloride (HZ), a tertiary amine—was studied *in vitro*, under conditions simulating those found in the stomach, to determine if nitrosamines are formed. Gas chromatography–mass spectrometry was used to monitor the latter compounds. We found that, in the presence of sodium nitrite, HZ undergoes oxidative cleavage and nitrosation, forming three nitrosamine compounds, *N*-(4-chlorophenyl)benzyl]-*N'*-nitrosopiperazine (A), *N*-[2-(2'-hydroxyethoxy)ethyl]-*N'*-nitrosopiperazine (B), and *N*, *N'*-dinitrosopiperazine (C). However, when ISDN (0.8 g) and HZ (2.0 g) were incubated together for 1 hr, only *N*-[α-(4-chlorophenyl)benzyl]-*N'*-nitrosopiperazine (A) was recovered. Although preparations of HZ contain (A) as an impurity, the quantity is trivial (0.5 ng/mg drug), and the bulk of the material detected is formed by interaction of ISDN with HZ. Because some individuals may ingest isosorbide dinitrate and hydroxyzine HCl, or analogous combinations, over a period of years, the risk posed by this type of drug interaction should be determined.

Chemical substances in the environment are increasingly recognized as causative agents in human cancers [1]. Communities in which large quantities of nitrites and/or nitrosatable compounds are present in food and water have high incidences of gastrointestinal cancers [2,3]. When ingested alone, nitrites are relatively non-toxic, but in the presence of secondary or tertiary amines they may form nitrosamines [4]. Many nitrosamines in low concentrations are known to be carcinogenic in animals when administered over long periods of time [5–7].

Recently, we described a new type of drug–drug interaction under conditions found in the human stomach* whereby vasodilator drugs of the organic nitrate class generate nitrite ions. We have also shown that the concentrations of nitrites produced are sufficient to *N*-nitrosate propranolol,* [8] a drug which contains a secondary amine function. In contrast to their formation from secondary amines, the formation of nitrosamines from tertiary amines requires oxidative cleavage of the tertiary amine group by a nitrosonium ion prior to nitrosation. In the present communication, we report that interaction between isosorbide dinitrate and hydroxyzine HCl, a (bis)tertiary amine (Fig. 1), also results in the formation of a nitrosamine product.

MATERIALS AND METHODS

Reagents and chemicals

Hydroxyzine HCl (HZ) was a gift from Pfizer Laboratories, Brooklyn, NY. The pharmaceutical formulations of isosorbide dinitrate (ISDN) used in this study were 25:75 mixtures of isosorbide dinitrate and lactose, received as gifts from various manufacturers. Other chemicals were purchased from the following sources: $NaNO_2$, Mallinckrodt AR, Jersey City, NJ; HCl (analyzed), J. T. Baker Chemical Co., Phillipsburg, NJ; $ClCH_2CH_2OCH_2CH_2OH$ (99%) and $N(CH_2CH_3)_3$ (99%), Aldrich Chemical Co., Inc. Milwaukee, WI.

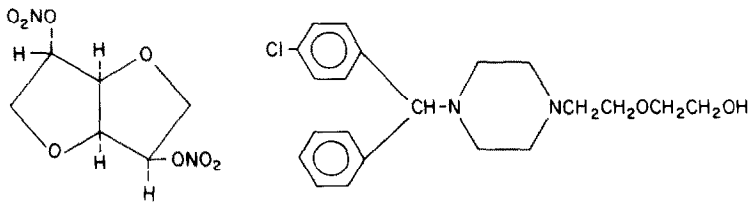
Detection of nitrosamines

Griess test. The qualitative Griess test was used to detect the presence of nitrosamines or nitrites, by the development of a violet–red color [9].

Extraction procedure. HZ was incubated for various periods of time with $NaNO_2$ or ISDN in 0.1 N HCl (100 ml). Following incubation, solutions were concentrated, adjusted to pH 3.4 with $NaHCO_3$, and extracted with benzene. Benzene was used as the extractant here because unreacted HZ, but not the nitrosamine product, is relatively insoluble in this solvent. The remaining aqueous phase then was adjusted to pH 8.0 and extracted with ethyl acetate, and both the aqueous and ethyl acetate phases were retained for further study. When HZ was incubated with ISDN, any of the latter which did not react was removed by sublimation from the extracted material prior to analysis by gas chromatography–mass spectrometry (g.c.–m.s.). Recovery of nitrosamines by this method was 30 per cent of the quantity of nitrosamine originally present in the incubation mixture, as determined by comparison with standard solutions of the authentic compounds.

Thin-layer chromatography. The dried materials from the organic solvent and aqueous extracts were dissolved in CH_2Cl_2 and applied to 2.5 × 10 cm thin-layer chromatographic plates coated with silica gel, 250 μ thick (Analtech, Inc., Newark, DE).

* I. H. Raisfeld, C. Lin and L. L. Chung, manuscript submitted for publication.



ISOSORBIDE DINITRATE HYDROXYZINE

Fig. 1. Structure of ISDN (left) and of HZ (right).

The material from the organic phase was developed for 15 min in $CHCl_3$:ligroin (2:1) and material obtained from the aqueous phase was developed in $CHCl_3$:EtOH (99:1). Compounds were detected by ultraviolet light. In the initial studies, every u.v.-absorbing spot was recovered, extracted with $CHCl_3$ or CH_2Cl_2, and subjected to the Griess test. Compounds that gave a positive result were studied further.

For preparative t.l.c., dried residues of the extracts were dissolved in CH_2Cl_2 and applied to 20 cm × 20 cm plates coated with 1000 μ silica gel (Analtech, Inc.). The material from the benzene extracts was developed in $CHCl_3$:ligroin (2:1), and ethyl acetate-extracted materials were developed in $CHCl_3$. For g.c.–m.s. studies the compounds were dissolved in CH_2Cl_2 or toluene.

Gas chromatography–mass spectrometry. Samples were injected at 250° into a Perkin–Elmer F42 gas chromatograph fitted with conditioned (72 hr) 6 ft glass columns packed with 3% OV 17, and were detected by an N-P detector at 300°, bead current 570, using N_2 as a carrier gas, 30 ml/min.

Nuclear magnetic resonance spectrometry

Nuclear magnetic resonance spectra were obtained from a Varian EM-360 60 MHz nuclear magnetic resonance spectrometer at 25° using $CdCl_3$ as the solvent and tetramethylsilane as the internal standard.

Synthesis of nitrosamines

N-[α-(4-chlorophenyl)benzyl] - N' - nitrosopiperazine (A). To *N*-[α-(4-chlorophenyl)benzyl]-piperazine (1.6 mmoles) and N HCl (3.2 mmoles), $NaNO_2$ (1.7 mmoles) was added. The solution was adjusted to pH 8.0 with $KHCO_3$, extracted with ether, and evaporated to give a sticky yellow residue. Recrystallization of this material from ether–ligroin yielded 0.25 g (50 per cent yield) of the desired *N*-[α-(4-chlorophenyl)benzyl]-*N'*-nitrosopiperazine, m.p. 122°, mol. wt. 316.81 by mass spectral analysis.

N-[2-(2'-hydroxyethoxy)ethyl] - N'- nitrosopiperazine (B). This was prepared from *N*-ethoxycarbonylpiperazine, which had been synthesized by the procedure of Moore *et al* [10]. A solution of *N*-ethoxycarbonylpiperazine (10.0 mmoles), β-chloroethyl-β-hydroxyethoxy ether (20 mmoles), and triethylamine (11.0 mmoles) in toluene was extracted with N HCl, and the aqueous extract was neutralized with potassium carbonate, extracted with CH_2Cl_2, and evaporated to dryness. The residue was vacuum distilled at 130° (0.1 mm Hg) and yielded 1.71 g (70 per cent yield) of *N*-[2-(2'-hydroxyethoxy)ethyl]-*N'*-ethoxycarbonylpiperazine, mol. wt 246. The structure was confirmed by ^{1}H n.m.r. analysis and elemental analysis.

N-[2-(2'-Hydroxyethoxy)ethoxy)ethyl]piperazine was prepared from the above compound by base hydrolysis. *N*-[2'-(2-Hydroxyethoxy)ethyl]-*N*-ethoxycarbonylpiperazine (2.0 mmoles) and N NaOH (6.0 mmoles) were heated under reflux. The solution was neutralized with N HCl and dried. The residue was extracted with EtOH, and the alcohol-extracted residue was distilled under vacuum at 110–113° (0.02 mm Hg). The desired piperazine derivative (0.21 g) was recovered.

The latter was nitrosated by the addition of potassium nitrite (27 mmoles) to *N*-[2-(2'-hydroxyethoxy)ethyl]piperazine (13.5 mmoles) in N HCl. The mixture was adjusted to pH 7.5 with $NaHCO_3$, and the dried residue was extracted with ethyl acetate. Evaporation of the ethyl acetate produced a sticky yellow liquid. Preparative t.l.c. of this material on silical gel, CH_2Cl_2–EtOH (92.0:8.0) then gave 2.69 g (13.1 mmoles, 98 per cent yield) of *N*-[2-(2'-hydroxyethoxy)ethyl]-*N'*-nitrosopiperazine, mol. wt 203.

Dinitrosopiperazine (C), mol. wt 144, was prepared according to published procedures [11,12].

Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN.

RESULTS

Generation of nitrosamines from hydroxyzine and sodium nitrite

Hydroxyzine HCl (1.0 g) was incubated with $NaNO_2$ (0.5 g) for 3 hr at 37°. The incubation mixture was extracted with benzene and ethyl acetate; the materials which were isolated from the extracts were chromatographed on thin-layer plates. After t.l.c., two benzene-extracted compounds (R_f: 0.74 and 0.46) and one ethyl acetate-extracted compound (R_f: 0.35) gave positive Griess reactions. After preparative t.l.c., these compounds were analyzed by gas chromatography–mass spectrometry.

The compound which had an R_f of 0.74 displayed a small parent ion peak at *m/e* 315 and an intense peak at 285 which suggests the loss of a nitroso group. This pattern is consistent with the structure

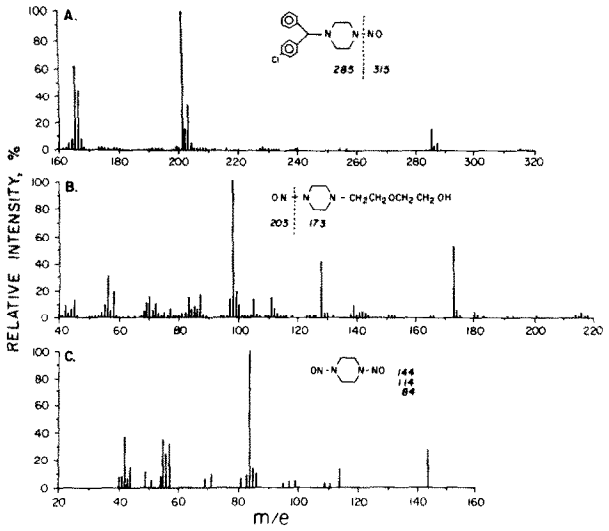


Fig. 2. Mass spectra obtained from a mixture of HZ (1.0 g) and $NaNO_2$ (0.5 g) following incubation for 3 hr at 37°. Panel A: *N*-[α-(4-chlorophenyl)benzyl]-*N'*-nitrosopiperazine (A); column-OV 17 (3%) oven temperature 200–250°; retention time of the large peak, 7.8 min. Panel B: *N*-[2-(2'-hydroxyethoxy)ethyl]*N'*-nitrosopiperazine (B); column-SE 30 (3%); oven temperature, 150–250°; retention time, 2.6 min, for the single large peak. Panel C: dinitrosopiperazine (C); column-OV 17 (3%); oven temperature, 100–250°; retention time, 6.1 min, narrow large peak.

of *N*-[α-(4-chlorophenyl)benzyl]-*N'*-nitrosopiperazine (A) (Fig. 2A). The compound obtained from the aqueous phase displayed an intense peak at 173, which is consistent with the loss of a nitroso group from *N*-[2-(2'-hydroxyethoxy)ethyl-*N'*nitrosopiperazine (B) (Fig. 2B). The third compound displayed a parent ion peak at 144, a peak at 114 reflecting the loss of one nitroso group, and an intense peak at 84 reflecting the loss of two nitroso groups. This evidence suggests the *N,N'*-dinitrosopiperazine structure (Fig. 2C).

To confirm this evidence, the physical properties of the reaction products were compared to those of authentic standards. The benzhydryl and hydroxyethoxyethyl nitrosopiperazine (A and B respectively) have not been described previously, and were prepared and identified (Table 1) as described under Materials and Methods. The R_f values of the synthetic nitrosamines (0.74, 0.35 and 0.46) and their mass spectra (Fig. 2 and Table 1) were identical to those observed with the products obtained from the reaction of hydroxyzine with $NaNO_2$.

Nitrosamine formation from hydroxyzine HCl *and isosorbide dinitrate*

HZ (2.0 g) and ISDN (0.8 g) were dissolved in 0.1 N HCl (100 ml) and incubated for 1, 3 and 24 hr at 37°. Organic and aqueous extracts were prepared as described above. The benzene extracts were dried, dissolved in toluene, and injected into the g.c.–mass spectrometer onto an SE-30 (3%) column, oven temperature 120–300°, 8°/min. At 10.8 min, a material indicated by the peak shown in Fig. 3 was eluted. The mass spectra for this compound and for

Table 1. Properties of synthetic nitrosamines

N*	Elemental analysis Calculated	Found	Nuclear magnetic resonance	Mass spectrum
A	%C: 64.66	64.74	1.18–2.70 (4H, multiplet)	Parent:315
	%H: 5.75	5.79	3.62–3.80 (2H, multiplet)	Intense peak 285
	%N: 13.31	13.47	4.10–4.32 (3H, multiplet)	(loss of NO)
			7.32 (9H, singlet)	
B	%C: 47.28	47.05	2.33–2.90 (6H, multiplet)	No parent peak
	%H: 8.45	8.60	3.20–4.40 (11H, multiplet)	Intense peak at 173
	%N: 20.68	20.45		(loss of NO)
C	None		3.78–4.18 (4H, multiplet)	Parent: 144
			4.28–4.50 (4H, multiplet)	Peak at 114
				Intense peak 84
				(loss of one
				or both NO)

* Nitrosamine A: *N*-[α-(4-chlorophenyl)benzyl]-*N'*-nitrosopiperazine; B: *N*-[2-(2'-hydroxyethoxy)ethyl]-*N'*-nitrosopiperazine; and C: dinitrosopiperazine.

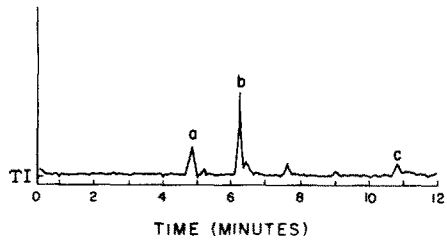


Fig. 3. Gas chromatogram of benzene extract of ISDN-HZ incubated for 24 hr. ISDN was removed by sublimation. Peak a is ISDN, peak b is diphenylketone, peak c is *N*-[α-(4-chlorophenyl)-benzyl]-*N'*-nitrosopiperazine (A).

chemically pure *N*-[α-(4-chlorophenyl)benzyl]-*N'*-nitrosopiperazine (A) are shown in Fig. 4. The other two possible nitrosamine compounds were not detected.

The amounts of *N*-[α-(4-chlorophenyl)benzyl]-*N'*-nitrosopiperazine, which were obtained following incubations for 1, 3 and 24 hr, were quantified by gas chromatography, at injection port and oven temperatures of 250°, on OV 17 (3%), 6 ft glass columns. With these conditions, the retention time of this compound was 11.3 min and the minimal detectable quantity was 2 ng. Known quantities of the chemically pure compound and residues from the benzene extracts were dissolved in toluene, and the peak heights were compared. The yields of nitrosamine obtained after 1, 3 and 24 hr of incubation were 22.3, 33.0 and 113.0 μg respectively.

Hydroxyzine HCl was dissolved in benzene in the absence of nitrate or nitrite. Small quantities of *N*-[α-(4-chlorophenyl)benzyl]-*N'*-nitrosopiperazine were consistently detected as an impurity, approximately 0.5 ng/mg of drug.

* I. H. Raisfeld, C. Lin and L. L. Chung, manuscript submitted for publication.

DISCUSSION

The major findings of this investigation are as follows: (a) hydroxyzine and nitrite interact *in vitro* in acidic solutions to produce three nitrosamines, and (b) two drugs in clinical use, isosorbide dinitrate and hydroxyzine, interact *in vitro* at pH 1.0 to generate a nitrosamine product.

We reported recently that, with conditions found in the human stomach, organic nitrate vasodilator drugs, including isosorbide dinitrate, are hydrolyzed to produce small quantities of nitrite.* Sufficient nitrite is produced from therapeutic doses of these drugs, so that nitrosation of a secondary amine drug, such as propranolol, occurs [8].

Nitrosamine formation from tertiary amines is considered to be a more complex reaction than nitrosation of secondary amines because the process of nitrosamine formation involves oxidative cleavage of the tertiary amine to form a secondary amine, which is then nitrosated [13,14]. Three nitrosamines form from the interaction of hydroxyzine and sodium nitrite. One of these, dinitrosopiperazine (C), is known to induce cancer of the nose, esophagus, liver and lung in laboratory animals [5, 6, 15]. The other two nitrosopiperazines, (A) and (B), have not been tested for carcinogenicity; however, many structurally similar nitrosamines are carcinogens [5–7].

Formation of a nitrosamine from the interaction of a vasodilator drug of the nitrate class and a tertiary amine drug has not been described previously. The quantities of the nitrosamine, recovered following incubation of hydroxyzine HCl and isosorbide dinitrate for 3 hr (33.0 μg) and 24 hr (113 μg), may be clinically relevant since preparations of ISDN are formulated to release drug in the GI tract over a protracted period of time. Usually, the human stomach empties within 1 hr. Following incubation of hydroxyzine and ISDN for 1 hr at 37°, 22.3 μg of *N*-[α- (4-chlorophenyl)benzyl] - *N'*-nitrosopiperazine (A) were recovered. The yield of nitrosamine is approximately 10 ng/mg of hydroxyzine. The quantities of the drugs used for this experiment, 2 g

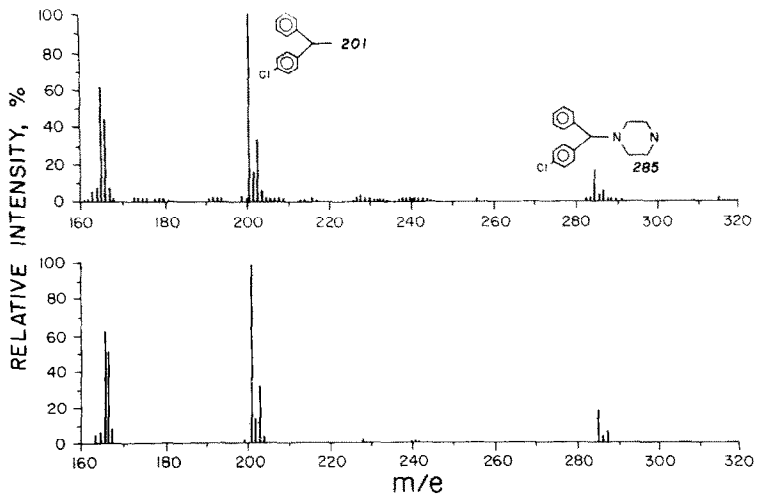


Fig. 4. Mass spectrum of synthetic *N*-[α-(4-chlorophenyl)benzyl]-*N'*-nitrosopiperazine (top panel). Mass spectrum of peak eluted at 10.8 min (Fig. 3) from benzene extract of ISDN–HZ mixture (bottom panel).

hydroxyzine and 800 mg isosorbide dinitrate, are equivalent to forty times the average single dose likely to be taken by a patient and ten times the total daily dose. If this reaction occurs with similar stoichiometry in the stomach, then 0.5 μg of this nitrosamine may form each time a patient ingests 50 mg hydroxyzine with 40 mg isosorbide dinitrate.

Hydroxyzine is commonly taken by patients with cardiovascular disease for control of symptoms of anxiety. Many of these patients also take nitrate ester vasodilator drugs. Long-term survival of patients with cardiovascular disease is common, and these drugs may be taken together for many years. For this reason, it is important to investigate the carcinogenic potential of these reactions. We plan to administer these drugs to laboratory animals to determine whether these reactions occur under physiologic conditions. If nitrosamines are produced *in vitro,* formation of nitrosamines could, theoretically, be prevented while maintaining the utility of the drugs. Administration of nitrates as sublingual or topical preparations would prevent accumulation of high concentrations of nitrite in the stomach. Alternatively, if the drugs were administered orally, patients should be advised against simultaneous ingestion of nitrate and amine drugs. To insure against nitrosamine formation from amines found in common foodstuffs, organic nitrates could be formulated with ascorbic acid or similar agents that retard nitrite formation under acid conditions [16–19].

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REFERENCES

1. T. H. Maugh, *Science* **183**, 940 (1974).
2. G. N. Wogan and S. R. Tannenbaum, *Toxic. appl. Pharmac.* **31**, 375 (1975).
3. P. N. Magee, *Fd. Cosmet. Toxic.* **9**, 207 (1971).
4. C. L. Walters, *Chem. Br.* **13**, 140 (1977).
5. H. Druckery, S. Ivankovic, J. Bucheler, R. Preussman and C. Thomas, *Z. Krebsforsch* **71**, 1976 (1968).
6. P. N. Magee and J. M. Barnes, *Adv. Cancer Res.* **10**, 163 (1967).
7. J. Sander and F. Schweinsberg, *Zentbl. Bakt. Hyg. I Abt.* (Orig. B) **156**, 299 (1972).
8. I. Raisfeld and J. Cheng, *Fedn Proc.* **38**, 2385 (1979).
9. F. Feigl and V. Anger, in *Spot Tests in Organic Analysis*, 7th Edn. p. 293. Elsevier, Amsterdam (1966).
10. T. S. Moore, M. Boyle and T. Thorn, *J. chem. Soc.* 39 (1929).
11. A. Ladenberg, *Ber. dt. Chem. Ges.* **24**, 2400 (1891).
12. H. J. Backer, *Recl. Trav. chim. Pays-Bas Belg.* **32**, 39 (1913).
13. P. A. S. Smith and H. G. Pars, *J. org. Chem.* **24**, 1324 (1959).
14. W. Lijinsky, L. Keefer, S. Conrad and R. Van de Bogart, *J. natn. Cancer Inst.* **49**, 1239 (1972).
15. M. Rink and M. Mehta, *Naturwissenchaften* **48**, 51 (1961).
16. W. Lijinsky and M. Greenblatt, *Nature New Biol.* **236**, 177 (1972).
17. S. Mirvish, *Ann. N.Y. Acad. Sci.* **258**, 175 (1975).
18. D. Ziebarth and G. Scheunig, in *Environmental N-Nitroso Compounds Analysis and Formation* (Eds. E. A. Walker, P. Bogovski and L. Griaute), p. 279. IARC 14, Lyon (1976).
19. N. P. Sen and B. Donaldson, in *N-Nitroso Compounds in Environment* (Eds. P. Bogovski and E. A. Walker), p. 103. IARC 9, Lyon (1974).