

Volatile *N*-Nitrosamines in the Urine of Normal Donors and of Bladder Cancer Patients¹

Tadao Kakizoe,² Tusn-Tien Wang, Vincent W. S. Eng, Rudolf Furrer, Peter Dion, and W. Robert Bruce³

Ontario Cancer Institute, Toronto, Ontario, Canada

ABSTRACT

Volatile *N*-nitrosamines were detected in the urine of male volunteers with gas-liquid and high-pressure liquid chromatography interfaced to the thermal energy analyzer. Of 50 samples from normal males, 10 contained nitrosodimethylamine (0.02 to 0.10 $\mu\text{g/liter}$), 6 contained nitrosodiethylamine (0.02 to 3.10), 9 contained nitrosomorpholine (0.06 to 0.67), and none contained nitrosodibutylamine. Of 4 samples from bladder cancer patients, 2 contained nitrosodibutylamine (0.35 and 0.66). Cigarette smoking did not appear to be related to the pattern or amount of urinary volatile *N*-nitrosamines. The possibility that the *N*-nitrosamines arise from the diet or from endogenous production is considered.

INTRODUCTION

Human bladder cancer is associated with generalized mucosal changes involving all the organs covered with transitional epithelium (3), and it seems likely that it is a consequence of exposure of the mucosa to carcinogens in the urine. The identification of such carcinogens has been achieved in a small number of tumors which are associated with certain occupations, but the etiology of the majority of these tumors remains obscure. Many chemicals (16), including the nitrosamines, have been shown to be bladder carcinogens in experimental animals, but these chemicals have not been detected in the urine of patients with bladder cancer.

We have recently observed that the feces of normal volunteers frequently contain several nitrosamines (19). One of these compounds, NDBA⁴ (5), is known to be a strong bladder carcinogen in experimental animals. It was thus important to ascertain whether human urine contained nitrosamines and in particular to determine whether such nitrosamines included NDBA. To carry out these studies, we have used TEA (8) which, because of its high sensitivity and specificity, is an effective tool for detecting these compounds in $\mu\text{g/kg}$ concentrations.

MATERIALS AND METHODS

Apparatus. For GLC-TEA analysis, a single-column isothermal gas chromatograph with a stainless steel column (0.125 inch outer diameter x 14 ft long) packed with 3% OV-225 or 10% Carbowax 20 M on Chromosorb W, 80 to 100 mesh, was used. The column temperature was 150°, and the argon carrier gas had a flow rate of 35 ml/min. A TEA (Thermo Electron, Model TEA-502) was used as the detector of *N*-nitrosamines at attenuation $\times 8$ and a furnace temperature of 450°.

For HPLC separations or HPLC-TEA analysis, a high-pressure pump (Waters Associates, Model 6000A), an injector (Waters Associates, Model U6K), and μ Porasil column (0.39 x 30 cm) were used. The solvent system was 5% acetone-95% 2,3,4-trimethylpentane at a flow rate of 1 ml/min. The same TEA detector was used, also at attenuation of $\times 8$ and a furnace temperature of 500°.

Donors and Urine Samples. Normal urine samples were collected from 27 male donors, 10 smokers and 17 non-smokers. The donors were 24 to 75 years in age, were eating a western diet, were not taking any medications, and were free of any clinical evidence of urinary tract infection. The urine samples were collected in washed glass bottles in the evening and were refrigerated overnight. In some cases, multiple samples were collected from the same donors. The pH of all samples ranged from 6.0 to 7.0. In control studies, sodium ascorbate (1 mg/ml) was added to a fraction of some of the samples, either immediately after collection or on arrival of the sample at the laboratory. The presence of this compound, which inhibits the nitrosation of amines (1, 15) did not lead to a measurable reduction in the amount of nitrosamine observed. This result indicates that artifactual formation of *N*-nitrosamines during the extraction procedure and overnight storage was negligible.

Urine samples from 4 bladder cancer patients were examined by the same protocol. The samples were sterile as confirmed by urinary bacteriological culture and had a pH between 6.0 and 7.0. At the time of the collection, the patients were not taking medications.

The first patient was a 65-year-old white male who had undergone transurethral resection of the tumor at another hospital where the pathology reports revealed anaplastic transitional cell carcinoma. Repeat cystoscopy revealed a crater around the right ureteric orifice created by the previous operation. Most of the mucosal surface of the bladder was covered with carcinoma *in situ*, which possessed a velvety erythematous nodular appearance with no clearly defined margins. His previous history was remarkable only because he had been a heavy cigarette smoker who had smoked more than 50 cigarettes/day for more than 20 years. The second patient was a 67-year-old white male

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² Present address: Urology Division, National Cancer Center Hospital, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104, Japan.

³ To whom requests for reprints should be addressed, at the Ontario Cancer Institute, 500 Sherbourne Street, Toronto, Ontario, Canada, M4X 1K9.

⁴ The abbreviations used are: NDBA, *N*-nitrosodibutylamine; TEA, thermal energy analyzer; GLC, gas-liquid chromatography; HPLC, high-pressure liquid chromatography; DCM, dichloromethane; NDMA, *N*-nitrosodimethylamine; NDEA, *N*-nitrosodiethylamine; NMOR, *N*-nitrosomorpholine.

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who had been a cigarette smoker for more than 30 years and had transitional cell carcinoma asynchronously in both the renal pelvis and the bladder. Of the third and fourth patients, one was a smoker and one was not. Both had single, tiny papillary tumors. The patients continued their usual smoking habits during the period that they supplied the urine samples.

Extraction. Two hundred ml of each urine sample were filtered through a Buchner fine filter and were extracted 3 times with 20 ml of dichloromethane in the presence of 20 g of sodium chloride. The DCM was then dried over sodium sulfate, and the sodium sulfate was then washed 3 times with 20 ml of DCM. The combined DCM extracts were evaporated under a vacuum at 50° to a volume of 0.2 ml. This concentrated solution was used for the analysis. The recovery of volatile nitrosamines by this method was tested by "spiking" urine samples, devoid of detectable nitrosamines, with NDMA, NDEA, NDBA, and NMOR to a concentration of 1.0 µg/liter. The yield was 50, 58, 77, and 90%, respectively, as determined by the GLC-TEA method described above. GLC-TEA analysis of the reagents used and of washings from glassware showed them to be free of volatile nitrosamines.

RESULTS

Typical patterns observed when a urine extract from a normal donor was applied to the GLC-TEA are illustrated in Chart 1. In Chart 1a there is a peak corresponding to the retention time for NDMA as well as one for an unidentified compound. Samples taken from the same donor on different days sometimes showed considerable differences in these patterns, and a variety of peaks corresponding to the retention times of a number of known *N*-nitrosamines were occasionally detected (Chart 1b). Since the TEA is highly specific for nitroso compounds, these data suggested that

human urine may contain a number of volatile nitrosamines.

Additional support for the assignment of the GLC-TEA peaks as volatile nitrosamines was provided by the HPLC. It was shown that the HPLC when interfaced to the TEA provided a similar separation of the TEA-responsive peaks. For instance, the sample used in Chart 1a gave rise to a peak with the same retention time as that for NDMA on the HPLC-TEA as it had shown on the GLC-TEA. Repeated injections of this urine sample were made on the HPLC, and the fraction corresponding to the retention time of NDMA was collected and combined. The solvent was partially evaporated with a nitrogen stream, and the remaining sample was applied to the GLC-TEA. The tracing that resulted showed a single peak corresponding to the retention time of NDMA. This same procedure, HPLC separation followed by GLC-TEA detection, was carried out with urine samples which had HPLC peaks corresponding to NDEA and NMOR with the corresponding results on GLC-TEA. These results thus show that the 3 compounds giving the TEA responses have the same retention times on GLC and HPLC as do the 3 nitroso compounds NDMA, NDEA, and NMOR.

Further evidence for the presence of volatile nitrosamines was provided by the response of these compounds to HBr. It is known that *N*-nitrosamines are sensitive to mild reduction and that, in the presence of HBr, they can lose the nitroso group (6). A portion of a sample which gave rise to multiple TEA peaks was therefore treated with HBr and acetic acid. When the treated sample was applied to the GLC-TEA, it gave no peaks. These results, taken together, with the unequivocal demonstration of nitrosamines in feces (19), as cited below, provide strong evidence for the presence of volatile nitrosamines in human urine.

In order to gain a perspective on the levels of the volatile nitrosamines in urine, specimens were obtained from 27 donors, some of whom were smokers and others who were

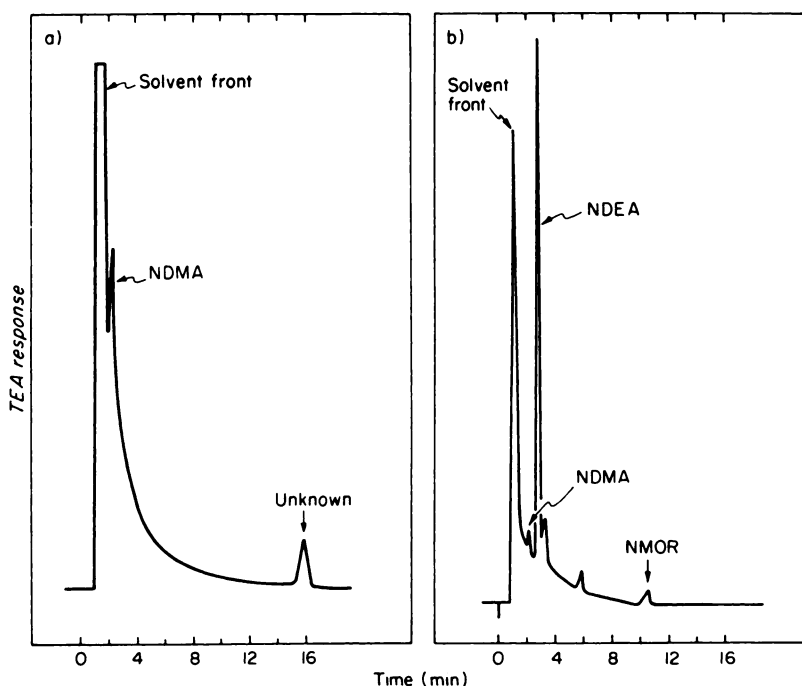


Chart 1. a, typical and frequently seen pattern of volatile *N*-nitrosamines on GLC-TEA analysis. NDMA and unknown nitroso peak are seen. TEA attenuation was $\times 8$. In b, a variety of peaks of *N*-nitrosamines were occasionally detected. A pattern from a smoker (Table 1, Column 1, E) analyzed by GLC-TEA is illustrated. TEA attenuation was $\times 8$.

not. The amounts found in each specimen are shown in Table 1. No nitrosamines were detected in 11 of 22 samples from smokers and 20 of 28 samples from nonsmokers. In the smokers, 6 of 22 samples contained NDMA, in the range 0.03 to 0.10 $\mu\text{g/liter}$; NDEA was detected in 3 of 22 samples in the range 0.09 to 3.10 $\mu\text{g/liter}$; and NMOR was found in 5 of 22 samples at the level of 0.07 to 0.67 $\mu\text{g/liter}$. In the nonsmokers, 4 of 28 samples contained NDMA, in the range 0.02 to 0.10 $\mu\text{g/liter}$; NDEA was detected in 3 of 28 samples between 0.02 and 0.05 $\mu\text{g/liter}$; and NMOR was detected in 4 of 28 samples between 0.06 and 0.17 $\mu\text{g/liter}$. No NDBA was found in any of the samples from normal donors.

The patterns observed when urine extracts from bladder cancer patients were applied to the GLC-TEA also showed considerable variation. Chart 2 shows the pattern observed for the first patient. In this case, 7 peaks were seen. Two of these, labeled NDMA and NDBA, were found to have the

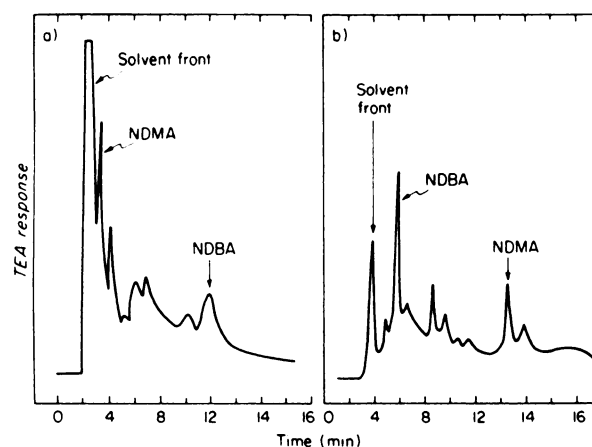


Chart 2. a, gas-liquid chromatogram for a 10- μl DCM extract of the urine from the first bladder cancer patient. TEA attenuation was $\times 8$. b, high-pressure liquid chromatogram from a 10- μl DCM extract of the urine from the first bladder cancer patient. TEA attenuation was $\times 8$. Both NDBA and NDMA, in samples and standards, had small shoulders following the main peaks on HPLC-TEA. The peak order of NDBA and NDMA is reversed in GLC-TEA and HPLC-TEA.

Table 1

Volatile nitrosamines in human urine

After correction with recovery efficiency as shown in text.

| Normal donor | Concentration ($\mu\text{g/liter}$) | | | |
|---|---------------------------------------|------|------|------|
| | NDMA | NDEA | NDBA | NMOR |
| Nonsmoker | | | | |
| A | — ^a | — | — | 0.17 |
| B | — | — | — | — |
| C | — | 0.05 | — | — |
| D | — | 0.02 | — | — |
| E | — | — | — | 0.08 |
| F | 0.04 | — | — | 0.07 |
| G | — | — | — | — |
| H(3), ^b I, J, K, L, M, N, O, P, Q | 0.02 | — | — | — |
| | 0.10 | 0.02 | — | 0.06 |
| Smoker | | | | |
| A | — | 0.09 | — | 0.07 |
| B | — | — | — | 0.11 |
| C | 0.03 | — | — | — |
| | 0.09 | — | — | — |
| D | 0.03 | — | — | — |
| | — | — | — | 0.08 |
| E | 0.10 | — | — | — |
| | 0.03 | — | — | — |
| F | — | — | — | — |
| | 0.03 | — | — | — |
| G | — | — | — | — |
| | — | 3.10 | — | 0.67 |
| | — | 0.52 | — | 0.11 |
| F(3), G, H, I, J | — | — | — | — |
| Bladder Cancer Patient Donor | | | | |
| A | 0.32 | — | 0.35 | — |
| B | 0.70 | — | 0.66 | — |
| C, D | — | — | — | — |

^a —, not detectable ($<0.01 \mu\text{g/liter}$).

^b Number in parentheses, samples from same donor.

same retention time as the standards in both GLC and HPLC when the methods described above were used. The second bladder cancer patient also had both NDMA and NDBA present, while the urine of the third and fourth patients had no nitrosamines present in their samples. The amounts found in each specimen are shown in Table 1. In particular, the NDBA levels in the bladder patients were 0.35 and 0.66 $\mu\text{g/liter}$.

DISCUSSION

The studies presented indicate that the urine of normal individuals and bladder cancer patients can contain volatile N-nitroso compounds in concentrations as high as 3 $\mu\text{g/liter}$. The studies also suggest that the levels of these compounds in urine can vary greatly from individual to individual from day to day. The limited studies with cancer patients suggest further that urine may contain a known bladder carcinogen, NDBA. What is the source of these nitrosamines? Could they be of importance in the causation of cancer?

Several possible sources for these compounds might be suggested: smoking (2); urinary infection (11, 12); medication (14); diet (9); and endogenous formation (7). Smoking appears not to be associated with urinary nitrosamines because the pattern of NDMA, NDEA, and NMOR was essentially similar for both smokers and nonsmokers. The highest concentration of NDEA in a smoker's urine (Table 1, Column 1, E) was indeed obtained from this individual during a period when he had abstained from smoking for a period of 2 weeks. The formation of N-nitrosamines in infected urine has also been reported (11, 12). However, the donors in this experiment had no clinical evidence indicating urinary tract infection. It has also been reported that a variety of commonly used drugs, such as oxytetracyclines and aminopyrine, react very rapidly with nitrous acid to form NDMA (14). Since our donors were not taking medications when the samples were collected, the possibility of this kind of formation of N-nitrosamines seems unlikely.

Foods seem a more likely source of the nitrosamines in urine. Preformed nitrosamines in cured and uncured foods have been demonstrated (9) and it has been calculated that the likely intake of dialkyl and heterocyclic nitrosamines per person on a normal English diet is approximately 1 and 3 $\mu\text{g}/\text{week}$, respectively. NDMA and NDEA have been observed in the blood within 200 min after the ingestion of cooked bacon and spinach (7). In this case, it seems most likely that the compounds were produced in the body endogenously. The precursors are present in the gastrointestinal tract. Nitrite has been detected in the mouth (18), in the stomach, in the ileum, and in the colon (17). Indeed, volatile *N*-nitrosamines, including NDBA, have been unambiguously detected in the feces of normal donors (19), and the amounts at this site are, in general, higher than the levels in the urine. It thus seems most likely that the source of the urinary compounds might be attributed to endogenous formation in the gastrointestinal tract.

While it is possible that the volatile nitrosamines are the cause of bladder cancer, the relationship may be difficult to establish. The level of the known bladder carcinogen, NDBA, is low and probably variable. The dose of this compound used to induce tumors in rats (5) is typically 200 $\mu\text{g}/\text{kg}$. Of course, the tumors in these animals develop in 200 to 300 days, which is a much shorter time than the exposures likely in humans. A second difficulty concerns metabolism. The concentrations of the proximal carcinogens, such as *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (5), are probably more important than those of the parent compound. The measurement of the concentration of such compounds poses a difficult task. A further difficulty is that the development of bladder cancer may depend on many cofactors, as well as the concentration of the carcinogen. Coffee drinking (4), artificial sweeteners (10), and protease in the urine (13) may all alter the bladder mucosa to make it more sensitive to prolonged exposures to NDBA or other carcinogens.

The demonstration of volatile nitrosamines in the urine thus does not by itself provide evidence for the origin of these compounds or of the hazard that they may present to the urinary tract. The methods we have described, however, are suitable for the study of larger populations. Such studies could be directed towards the relations between the diet and the levels of nitrosamines, as well as the metabolism and distribution of these compounds. They could also be used to determine whether there is an association between these compounds and human disease.

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