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ANALYSIS OF HUMAN BLOOD FOR VOLATILE N-NITROSAMINES BY GAS CHROMATOGRAPHY—CHEMILUMINESCENCE DETECTION

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SUMMARY

A method was developed to separate and measure trace levels of volatile N-nitrosamines (NAs) in human blood that either eliminated or accounted for in vitro artifactual formation of N-nitrosodimethylamine (NDMA) through the use of water blanks, added inhibitor (ascorbic acid) and added morpholine. The absolute minimum detectable limit was 8 pg; minimum level of reliable measurement was 0.05 μg/kg for a 20-g blood specimen. Recovery of NDMA from blood was 93 ± 5%. Coefficient of variation was 25%. Bloods from 242 people were analyzed for volatile NAs. NDMA was the only NA found. Positive specimens were presumptively confirmed by their non-detection after ultraviolet photolysis and/or mass spectrometry. This paper presents additional evidence that in vivo NA formation occurs.

INTRODUCTION

A method to separate, identify and quantitatively measure volatile N-nitros-

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amines (NAs) in human blood at the sub-ppb level was developed by Lakritz et al. in 1980 [1]. Other investigators, in earlier papers, had reported N-nitoso-dimethylamine (NDMA) in some human biological fluids [2–4], but the Lakritz paper represented the largest single group of data concerned with the appearance of NDMA in human blood. Subsequently, similar findings have been reported [5—11] while other investigators have found little or no NDMA in blood [12—15].

Because of these conflicting reports, there is a question concerning the origin of NDMA, a known animal carcinogen. Does NDMA, that has been reported to appear in blood, saliva [16–18], urine [3, 4] and gastrointestinal aspirates [5, 19] really reflect in vivo formation; or could its appearance be the result of an analytical artifact either from pre-formed NDMA present in the reagents, from blood collection apparati, from NDMA formed during analysis or from pre-formed NDMA present in the diet?

Both direct and indirect evidence for in vivo nitrosation has been presented by a number of investigators [19–24]. Certainly the requisite precursors are present in situ in many body fluids, and conditions for in vivo nitrosation have been demonstrated [9, 25]. The problem of artifactual NA formation has been addressed [26—32], but the question of whether NDMA is really present and why it is present remains especially pertinent because trace levels of NDMA have been reported in biological fluids by many investigators.

This paper presents a method of analysis that minimizes artifactual NA formation thus permitting a quantitative assay for NDMA in some biological fluids, and reports blood NDMA levels collected over a six-year period from 116 healthy adult volunteers and 126 hospitalized patients. It also examines the effects of three different sample treatments in an attempt to eliminate in vitro artifactual NA formation.

EXPERIMENTAL*

Reagents and procedures

All chemicals used in the analysis, unless otherwise stated, were from various manufacturers and were reagent grade or better. All reagents were tested for pre-formed NAs before use. A 300-ml sample of dichloromethane (DCM) (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) from each bottle used for analysis was concentrated to 0.5 ml, then tested to ensure absence of a positive thermal energy analysis (TEA) response (TEA is also the trade name for a commercial chemiluminescence detector [33, 34]). Double-deionized water was passed through an Organics Q cartridge (Millipore, Bedford, MA, U.S.A.), resulting in a water with electrical resistance of 18 MΩ. It was then filtered through a 0.45-μm filter before it was finally extracted twice with DCM (100 ml of DCM per l of water) prior to use. Hydrochloric acid (6 *M*) and sodium hydroxide (5 and 1 *M*) were prepared using this water, then extracted with DCM as indicated above. Barium hydroxide, sodium chloride, anhydrous

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*Caution: Nitrosamines are potentially dangerous carcinogens. Care must be exercised when handling these materials. See local regulations for required destruction or proper disposal of laboratory wastes.

sodium sulfate and ascorbic acid, after being found free from NAs, were used without further treatment. Morpholine was doubly distilled then assayed to ensure absence of its corresponding NA. An internal standard solution of N-nitrosomethylethylamine (NMEA) in DCM was assayed for any NDMA contamination. Disposable glass Pasteur pipettes used to transfer specimens were rinsed with deionized water to remove any possible traces of residual nitrite, since nitrite and occasionally NDMA have been reported as contaminants [35]. All glassware was soaked in approx. 6 *M* sulfuric acid for at least 2 h then washed in a commercial glassware washer with a soap that was tested and found free from pre-formed NAs. The testing of the soap was necessary since some commercial dishwashing compounds have been reported to contain low levels of some NAs [36]. Glassware was rinsed for 10 min with tap water, then 5 min with deionized water. All concentrator tubes, Kuderna Danish flasks and Snyder columns were additionally rinsed with DCM. Special attention was paid to fritted glass funnels by subjecting them to the above cleaning procedure and rinsing the funnels sequentially with 50 ml each of deionized water, methanol, acetone and DCM. These funnels were then dried in an oven at 110°C. All distillation flasks were soaked in chromic acid for 24 h, then washed as above; in addition they were rinsed with DCM and dried before use. Boiling chips were rinsed with acetone and DCM, then dried overnight in an oven at 150°C. All blood collection apparati including representative samples of collection tubes, selected at random, were tested to verify that NDMA or any other compound capable of giving a false positive TEA response was present. All NDMA standard solutions were stored in another building to insure against cross-contamination. The analytical work-ups were performed at a location different from where the NDMA was housed.

Sample collection

Our previous experiments indicated that optimum sample size was 20 g [1]. Therefore all samples and water blanks were kept to 20 ± 5 g. All volunteers were instructed to fast from after their evening meal the day before blood was to be collected (ca. 12 h). They were permitted to drink water. Blood samples were collected by venipuncture from a peripheral vein in the patients arm using a disposable sterile needle and a 20-ml plastic syringe. The blood was immediately transferred to a sterile centrifuge tube made from virgin polypropylene that contained 35—50 mg of ascorbic acid to inhibit in vitro NA formation during storage. The specimen was shaken to disperse the ascorbate, then 1.00 ml of an aqueous solution of morpholine (2 mg/ml) was added to the specimen to measure the extent of nitrosation in the sample during storage and processing. The detection of nitrosomorpholine (NMOR) would indicate in vitro nitrosation. The specimen was then stored frozen at −20°C or below until ready for analysis.

Sample preparation

Distillation. A schematic flow diagram of the procedure is represented in Fig. 1. Frozen whole blood, serum or plasma was thawed overnight in a refrigerator, then mixed thoroughly by shaking. A known amount of the blood (corrected for the volume of the morpholine added) was placed in a 1-l distilla-

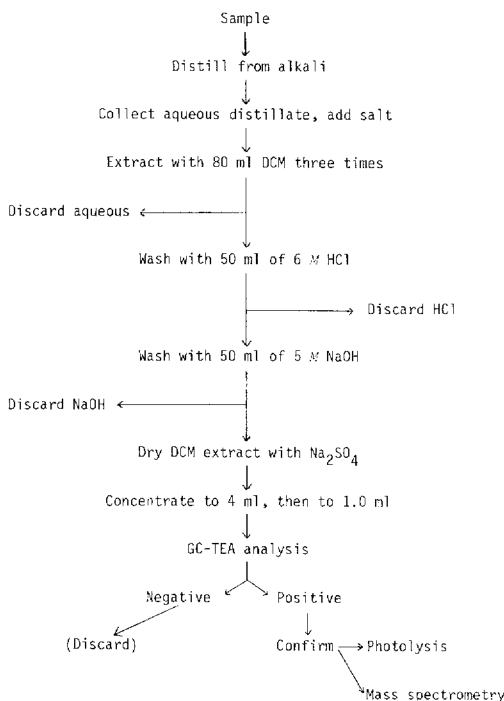

Fig. 1. Schematic flow diagram of procedure.

tion flask containing a few boiling chips, 20 ml of the "NA-free" water, 30 ml of 1 *M* sodium hydroxide, 8 g of barium hydroxide and an additional 35—50 mg of ascorbic acid. Only after appropriate testing to insure that the addition of another NA as an internal standard would not give rise to artifactual NDMA via its decomposition and subsequent transnitrosation, 1.00 ml of NMEA (0.02 mg/l in DCM) was added. The flask was set up for distillation at atmospheric pressure using standard tapered glass joints without any grease or rubber connections. The distillation flask was wrapped with aluminum foil to decrease heat loss and to avoid any photolysis of the NAs that might occur. The condenser was jacketed and maintained between 4 and 5°C. Foaming was avoided in most cases by beginning the distillation at a low variable transformer (Variac) setting of 50 V for the first 30 min, then increasing to 65V for the remainder of the time until the flask was almost dry. Total distillation time was about 2.5 h. A foam trap was an integral part of the distillation set-up (Part No. 517 000, Kontes, Vineland, NJ, U.S.A.).

The resulting aqueous distillate (pH > 10) was placed in a 250-ml separation funnel with 10 g of sodium chloride and extracted for 3 min with three separate 80-ml volumes of DCM. The DCM extracts were combined in a 500-ml separation funnel and washed, first with 50 ml of 6 *M* hydrochloric acid, then with 50 ml of 5 *M* sodium hydroxide. The resulting DCM extract (240 ml) was dried by passing through 35 g of pre-washed (with DCM) anhydrous sodium sulfate, held in a 60-ml coarse fritted glass funnel, and collected into a 500-ml Kuderna Danish flask fitted with a 4.0-ml concentrator tube. The volume was reduced to about 4.0 ml on a steam bath. Further reduction of the extract to

1.0 ml was achieved using a Snyder microcolumn and a 65°C water bath.

Dry column. A glass column (350 mm × 32 mm I.D. with a 60 mm × 6 mm drip tip) packed with Celite 545, sodium sulfate and hydrochloric acid was prepared. A sample of whole blood was divided into two equal volumes. One was passed through the column using a modification of the procedure of Pensabene et al. [37] employing hydrochloric acid instead of phosphoric acid. The other was distilled as noted above. Both extracts were assayed for NDMA.

Analysis and confirmation

An aliquot (7–9 μl) of the concentrated DCM extract was injected into a gas chromatography (GC) apparatus (Varian-Aerograph Model 1720, Palo Alto, CA, U.S.A.) interfaced with a thermal energy analyzer (Model 502, Thermo Electron, Waltham, MA, U.S.A.). The conditions for GC–TEA analysis are summarized. The column was 2.7 m × 3.2 mm I.D. stainless steel packed with 15% Carbowax 20M-TPA on 60–80 mesh Gas Chrom P. Helium at 42 ml/min was the carrier gas. Injector temperature was 180°C; column oven was programmed from 110 to 220°C at 4°C/min. When only NDMA and NMEA analysis was required, the GC column temperature was 140°C isothermal. Further details of this procedure have been reported [38]. Peaks appearing on chromatograms were compared to those of known standards, identified by retention times relative to the internal standard of NMEA, with quantitation by peak-height comparison to known calibration standards run at various times during the day.

Samples that indicated the presence of NAs were confirmed by a photolytic technique [39]. When sufficient NA was present (> 100 ng), it was confirmed by gas chromatography–mass spectrometry (GC–MS) using a Hewlett-Packard Model 5992B low-resolution quadrupole GC–MS system. The GC–MS instrument conditions are summarized. A glass capillary column, 30 m × 0.5 mm I.D., coated with UCON 5100, was used for all separations. Helium at 3.5 ml/min was the carrier gas. Injector temperature was 150°C; column temperature was 20°C for 2 min, programmed at 8°C/min to 160°C and held at this temperature. Three ions were monitored under the following conditions; electron energy 70 eV, electron multiplier 2000–2600. Integration time was 50 ms per mass monitored. Detailed description of the method has been reported [40]. Most MS for NDMA was low-resolution multiple-ion monitoring of ions of m/z 30, 42 and 74, but some pooled specimens were subjected to high-resolution MS (HRMS) as well. Both non-photolyzed and photolyzed samples were run.

As a further aid to confirmation and to compare some NDMA levels obtained by GC–TEA to levels obtained by GC–HRMS, fourteen randomly selected samples were distilled as noted. An aliquot of each sample was assayed for NDMA by GC–TEA; then another aliquot from the same sample was reassayed for NDMA by GC–HRMS [15].

RESULTS AND DISCUSSION

The analysis for NDMA in blood was reproducible at the sub-ppb level. Replicate analyses for NDMA using two different blood samples (A and B)

were performed. Analyses of ten identical aliquots of blood A showed the mean NDMA concentration to be 0.30 ± 0.08 μg/kg (1 S.D.). Similar analyses of eight identical aliquots of blood B showed the mean NDMA level to be 0.09 ± 0.02 μg/kg. Fig. 2 shows a representative chromatogram of a 9-μl injection of a distilled blood extract in DCM containing 0.07 μg/kg NDMA. Our criteria for positive identification as a signal-to-noise ratio > 2, although most of the samples exhibited a signal-to-noise ratio > 4. The minimum detectable limit in a 20-g blood specimen was 0.05 μg/kg (ppb). Below this level the signal-to-noise ratio was too low to permit positive detection. Our methodology could not distinguish between 0.00 (absolutely no response) and peaks less than 0.05 μg/kg, thus for calculations, all blood levels below 0.05 μg/kg were treated as 0.025. Using this as an estimate of the actual level instead of 0.00 made no significant difference in the mean or standard deviation and avoided the use of 0.00, making logarithmic and exponential transforms of the data possible.

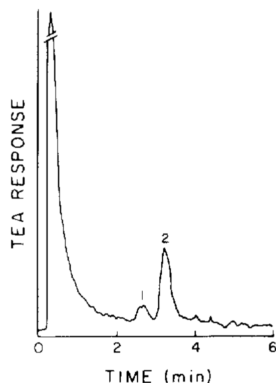

Fig. 2. GC—TEA profile of a blood sample containing 0.07 μg/kg NDMA after distillation, extraction into dichloromethane and concentration to 1.00 ml. Conditions: 9.0-μl injection, column temperature 140° C isothermal, attenuation × 8. Peaks: 1 = NDMA; 2 = NMEA, internal standard.

Recovery of NDMA from blood spiked at the 1.0—10 μg/kg level was 89.0 ± 7.5% (1 S.D.). An internal standard (NMEA) was not used to correct for possible losses during recovery experiments at the higher levels. Recovery experiments for NDMA at the 0.10—1.0 μg/kg level (low level) did incorporate an internal standard of NMEA and concentrations of NDMA were corrected for NMEA recovery. Recovery of NDMA from blood at this lower level was 93 ± 5%.

NDMA artifact formation by transnitrosation from possible decomposition of the NMEA internal standard was tested. Blood from one person was collected and divided into ten 20-g specimens. NMEA (1 ml, 0.02 μg/ml in DCM) was added to five test bloods, and 1.00 ml of DCM alone added to the others. They were paired and assayed for both NDMA and NMEA. Analysis of variance showed no difference between blood NDMA levels with or without added NMEA ($p < 0.05$). The variance ranged between 0.03 and 0.05 and was independent of added NMEA. Thus, transnitrosation was highly unlikely under the conditions of analysis. NMEA has been used successfully by us as an internal standard in over 300 biological specimens (blood, urine, gastric, duodenal and bile aspirates).

TABLE I

DRY COLUMN VERSUS DISTILLATION

Sample	Method	Recovery (%)	NDMA level* (μg/kg or ppb)
A	Dry column	60	2.8
A	Distillation	88	1.5
B	Dry column	68	0.47
B	Distillation	85	0.15
C	Dry column	68	0.66
C	Distillation	88	0.22
D	Dry column	62	0.63
D	Distillation	90	0.27
E	Dry column	68	0.51
E	Distillation	84	0.48
F	Dry column	65	0.53
F	Distillation	90	0.46

*NDMA was not corrected for NMEA recovery.

In an effort to shorten the sample preparation time and eliminate the need to distill the sample, two other preparation methods were examined that used (1) direct extraction and (2) a dry column. The direct extraction of the blood specimens with DCM was unsatisfactory owing to background compounds that coextracted and eluted with the solvent peak, completely obscuring the NDMA peak. A comparison between the distillation and the dry column showed a significant difference between the NDMA levels (uncorrected for recovery) from six paired samples ($p < 0.05$). Mean recovery of NMEA with dry column was 65 ± 3.5%; distillation was 88 ± 2.5 (Table I). The dry column method always gave higher NDMA levels and lower recoveries. There was concern that some artifactual formation of NDMA may have occurred because of the hydrochloric acid on the dry column as noted by Pensabene et al. [37].

Blood specimens from 116 healthy volunteers (68 males and 48 females with a mean age of 36 and no distinction made between smokers and non-smokers) were assayed for volatile NAs by the procedure incorporating the distillation. NDMA was detected in 80% of these specimens and is shown in Table II. The raw data were divided into two main groups: one in which no water blanks were analyzed (group 1) and one where water blanks were employed and the NDMA found in the water blank substracted from the specimen (group 2). The latter was further divided into (A) water blanks only, (B) water blanks and ascorbic acid and (C) water blanks, ascorbic acid and morpholine together. Single-factor analysis of variance indicated no significant difference among the three subgroups of the second set ($F = 1.05$), therefore the data from the three subgroups were combined. A significant difference between the two main groups (1 and 2) was noted ($p < 0.01$) indicating that the use of a water blank in group 2 was a critical factor.

NDMA levels as high as 0.8 μg/kg were detected in water obtained from our standard laboratory deionizer or single-stage still. Using the double-deionized 18-MΩ water, that in addition had been extracted with DCM, reduced the NDMA levels in the water substantially; however, NDMA occasionally appeared although at a much lower level (from none detected to 0.17 μg/kg). Whether

TABLE II

NDMA LEVELS IN BLOOD OF NORMAL HUMAN SUBJECTS

Values are expressed in μg/kg. In group 1 (37 males, 21 females, mean age 39 years) no water blanks were analyzed; in group 2 (31 males, 27 females, mean age 35 years) water blanks were employed.

	Group 1	Group 2
Mean NDMA level	0.67	0.18
S.D.	0.44	0.20
Standard error	0.06	0.03
Maximum level	1.9	0.75
Minimum level*	0.05	0.05

*Minimum confident detectable limit was 0.05 μg/kg. Some specimens gave no response at all and were considered as 0.025 for calculation purposes.

this appearance represented actual pre-formed NDMA in water or a nitrosating capacity of the water caused by dissolved NO_x which was able to combine with trace amines that could be concentrated during the analytical procedure, was very difficult to determine and impossible to control. Therefore a water blank had to be run along with each group of bloods distilled on any particular day, and the appropriate amount of NDMA subtracted to account for the amount in the water used in the analysis.

The role of ascorbic acid to inhibit amine nitrosation is well known. Mirvish et al. [41] showed that ascorbic acid was capable of blocking NA formation at pH 1—4 by competing with secondary amines for nitrite. Ascorbic acid was added to all our samples including water blanks. The level of ascorbic acid attained in our samples during storage was a minimum of 9.9 m*M*, an amount sufficient to react with any concentration of nitrite ion reported in normal blood. The addition of ascorbic acid to blood did not cause any significant difference in the mean blood NDMA levels as indicated by single-factor analysis of variance between subgroups of bloods in group 2, which was not surprising since the pH of the blood samples was between 7.2 and 7.7. However, reports of ascorbic acid inhibiting amine nitrosation at pH 7 [32] combined with our need to be consistent, since ascorbic acid was added to all our water specimens and gastric aspirates, prompts us to continue adding ascorbic acid.

The addition of morpholine, a readily nitrosatable amine (pK_a 8.7) having a rate constant 250 times greater than dimethylamine should also be an effective inhibitor acting as a preferentially nitrosated amine and a good indicator of artifactual in vitro nitrosation. NMOR was never detected in our blood samples from healthy individuals (detection limit of 0.2 μg/kg).

NDMA levels in pooled serum from hospital clinical laboratories are shown in Table III. The samples represent blood collected from an additional 126 different patients. Each of the 21 specimens was comprised of approximately equal volumes of serum from six individual patients selected at random whose only requirement was no evidence of kidney disease as determined by a normal serum creatinine, a measurement routinely performed on hospital patients' blood. No attempt was made to separate fasting from non-fasting, males from females or those persons taking medications. For this group standard glass

TABLE III

NDMA LEVELS IN SERUM FROM POOLED SAMPLES OF HOSPITALIZED PATIENTS

Identifier	NDMA level (μg/kg)	Identifier	NDMA level (μg/kg)
A	0.22	L	0.08
B	0.16	M	0.12
C	0.22	N	0.15
D	0.28	O	0.15
E	0.26	P	0.07
F	0.21	Q	0.07
G	0.25	R	0.07
H	0.20	S	0.10
I	0.29	T	0.11
J	0.30	U	0.24
K	0.09		

TABLE IV

GC—MS VERSUS GC—TEA: COMPARISON OF NDMA LEVELS IN IDENTICAL SAMPLES

Number	Sample description	NDMA level (pg/g)	
		GC—MS	GC—TEA
36-9	Water	52	44
55-1	Water	98	102
55-2	Water	720	576
55-3	Blood	77	N.R.*
44-6	Serum	250	187
55-20	Blood	350	318
41-1	Blood	800	703
55-10	Blood	560	748
36-8	Blood	219	223
36-7	Blood	263	242
45-1**	Blood	596	565
45-1	Blood	586	565
45-14	Blood	429	268
45-2	Duodenal	200	277
Mean injection volume (μl)		2.3	9.0

*N.R. = No response (less than 50 pg/g).
**45-1 was run twice.

vacuum type blood collection tubes with rubber stoppers were used. The mean level of NDMA in these pooled samples was similar to the mean level found in group 2 of the individual bloods from healthy volunteers. As a further aid to confirmation and to briefly compare two different detection methods, NDMA levels were measured in fourteen randomly selected specimens by the distillation procedure using two different detection methods: (1) GC—TEA and (2) GC—HRMS (Table IV). Analysis by log-log transform linear regression of the data showed no significant difference between the data obtained by the two methods ($p < 0.05$) suggesting that GC—HRMS as a routine method of

detection was not superior to GC—TEA, particularly when costs of equipment and analysis were considered. However, the value of HRMS should not be overlooked; it just may be unnecessary for many applications. It may be very valuable for applications of direct analysis of small samples or highly colored samples that cannot be confirmed by the UV photolysis procedure.

Confirmation of NDMA by photolysis was positive in 97% of the blood specimens as indicated by the disappearance of the NDMA peak after UV photolysis and reassay by GC—TEA. Fig. 3 shows a GC—TEA profile before and after photolysis. In a small number of samples a peak appeared close to the retention time for N-nitrosodipropylamine (NDPA) before photolysis. This peak did not disappear after 3 h of photolysis, suggesting that it was not an NA. Some non-NAs have been reported to give TEA responses [33, 42].

Fig. 4 shows a GC—low-resolution MS plot of three molecular ions for NDMA (74, 42 and 30) from a pooled blood sample before photolysis and the corresponding spectra after photolysis. There was complete disappearance of the peaks associated with NDMA after photolysis.

In addition to the fourteen previously noted samples that were confirmed by GC—HRMS, five bloods that were unable to be confirmed by the photolytic procedure owing to the dark color of the samples were confirmed to be positive by GC—HRMS.

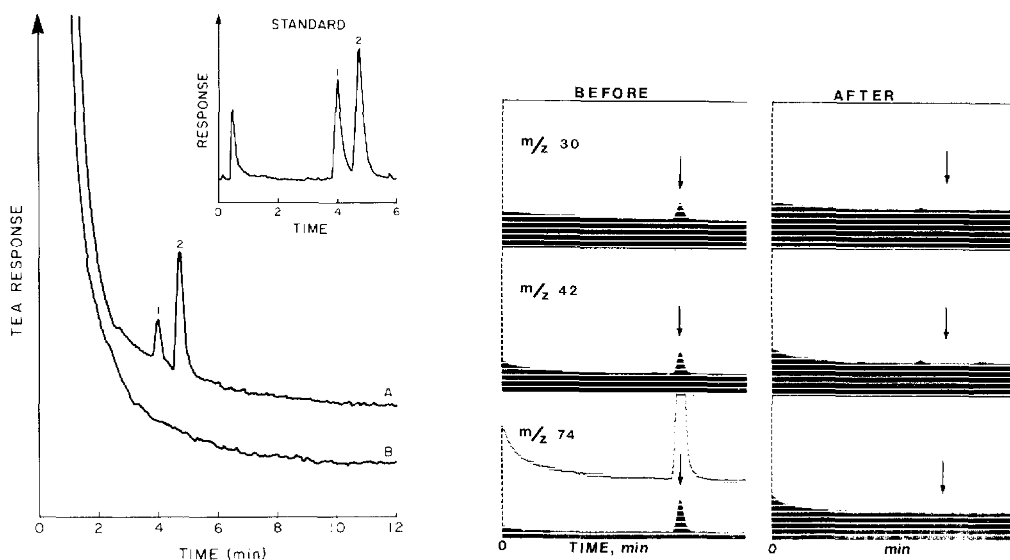

Fig. 3. GC—TEA profile of a 9.0-μl injection from a 20-g blood specimen. Conditions: temperature programme 120—220°C at 4°C/min, attenuation ×8. Line A is prior to photolysis; line B is same specimen after 3 h of UV photolysis (note complete disappearance of both nitrosamine peaks). Peaks: 1 = NDMA (0.17 μg/kg); 2 = NMEA, internal standard (representing 94% recovery). The inset shows pure standards under the same conditions for comparison.

Fig. 4. GC—low-resolution MS plot of three molecular ions (30, 42 and 74) for a pooled blood sample that gave a positive NDMA TEA peak before photolysis and the corresponding sample after UV photolysis.

CONCLUSIONS

The distillation method described shows that it is possible to analyze blood and other biological fluids for trace levels of volatile NAs [19]. The possibility of pre-formed NDMA from the diet has been significantly reduced by having the healthy, non-hospitalized people fast 12 h prior to blood collection. However, this fasting period may not completely preclude pre-formed NDMA detection since exposure can be other than dietary related.

The subtraction of the NDMA found in the water blank from the blood samples and the addition of NA inhibitors have eliminated or taken into account any measurable NDMA from artifactual formation. The necessity of a water blank is clearly reflected in the data collected, suggesting that previous data collected by us and other investigators without incorporating artifact control measures may have overestimated the apparent NDMA values. On the other hand, some who have reported the absence of NDMA in blood, may have simply used too small a sample. Many of our samples would have been negative had only 5 or 10 g of material been used.

While the information described in this paper does not completely preclude artifactual NA formation in blood, the detection and confirmation of low levels of NDMA adds additional support to the growing body of information that in vivo NA formation occurs. The relevance or extent of in vivo versus pre-formed NAs in the etiology of human cancer may, under certain pathological or environmental conditions, be an important one. Equally important may be the fact that humans without these certain pathological conditions may have the ability to detoxify NAs at low levels.

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