

N-NITROSAMINES—CONTAMINANTS IN BLOOD-COLLECTION TUBES

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Abstract—Volatile nitrosamines were found in commercially produced rubber-stoppered blood-collection tubes. Some rubber stoppers contained up to 147 ppb nitrosodimethylamine, 92 ppb nitrosodiethylamine and 1302 ppb nitrosomorpholine. These nitrosamines readily leached out of the stoppers to contaminate the tubes and their contents.

INTRODUCTION

Volatile nitrosamines, potent animal carcinogens, have been reported in human urine (Kakizoe, Wang, Eng, Furrer, Dion & Bruce, 1979), faeces (Wang, Kakizoe, Dion, Furrer, Varghese & Bruce, 1978) and blood (Fine, Ross, Rounbehler, Silvergleid & Song, 1977). During a recent study on nitrosamines in human blood (Lakritz, Simenhoff, Dunn & Fiddler, 1970), we observed unexpectedly high levels of *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA) and *N*-nitrosomorpholine (NMOR) in a blood sample. The latter compound has not previously been detected in significant quantities in biological samples or in foods. This report is the result of an investigation to determine whether the nitrosamines found were normally present in the blood or were artefacts introduced into the sample.

EXPERIMENTAL†

Materials. Dichloromethane (DCM), acetone and isooctane were obtained predistilled in glass from Burdick and Jackson (Muskegon, MI). Hydrochloric acid, sodium hydroxide and sodium sulphate (anhydrous) were ACS grade. All the reagents and distilled water used were checked and found to be free of nitrosamines.

Evacuated blood-collecting specimen tubes produced by four manufacturers (Kimble-Terumo, Elkton, MD; Jelco, Raritan, NJ; Becton-Dickinson & Co., Rutherford, NJ; Corning Glass Works, Corning, NY) were analysed for nitrosamines and amines. The tubes contained no additive and were silicone-coated or contained as an anticoagulant either sodium heparin, ethylenediaminetetraacetic acid (EDTA), sodium citrate, potassium oxalate or serum separator.

Methods. Rubber stoppers were removed from the blood-collection tubes and the exposed upper portion of each was removed to preclude the possibility of contamination from external sources. The lower portion of the stopper, which was in contact with the test tube, was immersed in 3 ml DCM for 30 min. The DCM was extracted three times with 3 ml 0.2 *N*-HCl, which in turn was re-extracted with an equal volume of DCM. The combined DCM extracts were dried by passage through anhydrous sodium sulphate and concentrated to 1.0 ml in a Kuderna-Danish apparatus on a steam bath and in a micro Snyder column in a water-bath at 68°C. The aqueous hydrochloric acid extracts, containing the free amines, were taken to dryness by lyophilization. The samples were reconstituted to 1.0 ml with water, and sufficient 0.2 *N*-NaOH solution was added to attain a pH of 10 prior to gas-chromatographic analysis.

The amines and nitrosamines inside the glass test tubes were removed by rinsing the tubes, including the additives, if any, with 3 × 3 ml 0.2 *N*-HCl and this acid solution was extracted with an equal quantity of DCM. The acid extract containing the amines and the DCM extract containing the nitrosamines were then concentrated as described above.

Analyses. The analysis for nitrosamines was accomplished by the use of a gas-liquid chromatograph (GLC) interfaced with a Thermal Energy Analyzer (TEA) Model 502 (Waltham, MA), which is extremely sensitive and specific for nitrosamines (Fine, Ruseh & Gunther, 1973). The nitrosamines were separated on a Varian-Aerograph Model 1720 gas chromatograph (Palo Alto, CA) containing a nickel column (9 ft × 1/8 in. OD) packed with 15% Carbowax 20 M-TPA on 60–80 mesh Gas Chrom P. The injector port temperature was 220°C, and the column temperature was programmed from 110 to 220°C at 4°C/min. With a helium flow rate of 42 ml/min, NDMA, NDEA and NMOR eluted at 4.2, 5.6 and 15.8 min, respectively. The TEA was operated under conditions similar to those reported by Fine & Rounbehler (1975). Samples containing concentrations as low as 0.1 ng/10 µl could be detected readily.

Apparent nitrosamines identified on the basis of GLC retention times and TEA detectability were confirmed by being exposed to ultraviolet light and then rechromatographed on the GLC-TEA (Duerr &

*Agricultural Research, Science and Education Administration, US Department of Agriculture. The products tested in this study were selected on the basis of availability. Reference to brand or firm name is given for the convenience of the reader and does not constitute endorsement by the US Department of Agriculture of any specific product over others.

†Note: Precaution should be exercised in the handling of nitrosamines since they are potential carcinogens.

Fiddler, 1977) to determine their photolability. Selected samples were further confirmed by separation and identification of the nitrosamines by high-pressure liquid chromatography (HPLC) interfaced with a TEA detector (HPLC-TEA). The nitrosamines were separated on a Laboratory Data Control HPLC equipped with a Constametric Pump II (Riviera Beach, FL) and a column (50 cm x 2.1 mm ID) packed with MicroPak Si 10 (10 µl) porous silica. The isocratic mobile phase was 5% acetone in isooctane operating at a flow rate of 1 ml/min. Elution times for NDEA, NDMA and NMOR were 6.3, 12.4 and 14.6 min respectively.

GLC-mass spectrometric analysis. For confirmation of the identity of the nitrosamines, a Varian-Aerograph Model 2700 GLC equipped with a glass column (6 ft x 1/4 in. OD) packed with 15% Carbowax 20M-TPA on Gas Chrom P was connected to a Varian MAT 311A mass spectrometer (MS; Varian Associates, Florham Park, NJ). The helium flow rate was 15 ml/min. The temperatures used for the detector, injector port and GLC-MS interface system were 200, 200 and 180°C, respectively, and the column was programmed from 90 to 140°C at 4°/min for NDMA and NDEA, and from 140 to 180°C at 6°/min for NMOR. The MS was operated in the peak-matching mode adjusted to a resolution of 1 in 10,000 or 12,000. The mass spectra were obtained at an ionizing voltage of 70 eV and an ion-source temperature of 150°C. The mass-to-charge ratios (*m/e*) of 74.04799 for NDMA and 102.07930 for NDEA were determined on the bases of the *m/e* 69.99857 and *m/e* 99.99361 perfluorokerosene reference peaks, respectively, by measuring the difference in *m/e*. The *m/e* of 116.05857 for NMOR was determined by using the *m/e* 106.07825 reference peak of xylene in a similar manner. The signal was recorded on both an oscilloscope and a recording oscilloscope.

Confirmation of the nitrosamines by MS required larger samples than those used for TEA analysis. For each analysis, the lower portions of ten stoppers were ground and extracted twice with 20 ml DCM, and the extract was passed through an acid Celite column and concentrated as described above.

The amines were quantitated by GLC on a Hewlett-Packard Model 5710A gas chromatograph (King of Prussia, PA) equipped with a nitrogen-phosphorus detector. Separation was achieved in a glass column (6 ft x 2 mm ID) packed with 12% Amine 220 plus 8% KOH on 100-120 Chromosorb W/AW (Supelco, Bellefonte, PA). The injector, column and detector temperatures were 200, 50 and 250°C, respectively, for all separations except morpholine, for which the column temperature was 80°C. Carrier-gas flow rate was 15 ml/min at all times. At a column temperature of 50°C, the retention times for the amines were (in min): trimethylamine 1.2, methylamine 1.6, dimethylamine (DMA) 1.8, ethylamine 2.5 and diethylamine (DEA) 5.2. At 80°C, morpholine (MOR) eluted at 12.5 min.

RESULTS

No nitrosamines were detected in any of the reagents or distilled water used in the analytical procedures. Therefore, attention was directed to the blood-collection tubes and their anticoagulant contents (Table 1). Tubes manufactured by Kimble-Terumo had no detectable nitrosamines when empty, but those containing sodium citrate in solution showed nitrosamines up to 16 ppb NDMA, 9 ppb NDEA and 143 ppb NMOR. When a solution of EDTA was present as the anticoagulant, levels up to 17 ppb NDMA, 5 ppb NDEA and 158 ppb NMOR were found. Neither the Jelco brand blood-collection tubes, empty or containing solid heparin, nor the Becton-Dickinson and Corning brand tubes, empty or containing anticoagulants, contained detectable nitrosamines.

The presence of nitrosamines only in tubes with liquid anticoagulants suggested the possibility of the rubber stoppers as a source of the nitrosamines. Results of the analyses of rubber stoppers from major brands of evacuated blood-collection tubes are shown in Table 2. In 17 of 20 Kimble-Terumo tubes, rubber stoppers contained 11-98 ppb NDMA, 8-67 ppb NDEA and 85-678 ppb NMOR. The Jelco brand stoppers, although no nitrosamines were detected in the tubes, contained 39-147 ppb NDMA, 22-92 ppb NDEA and 359-1302 ppb NMOR. None of the Becton-Dickinson brand stoppers contained detectable nitrosamines.

Table 1. Nitrosamines in the contents of blood-collection tubes

Manufacturer	Additive	Positive tubes (no./no. tested)	Nitrosamine levels (ppb)*		
			NDMA	NDEA	NMOR
Kimble-Terumot†	None	0/5	ND	ND	ND
	Na citrate (liquid)	4/5	5-16 (8)	3-9 (4.4)	61-143 (93)
	EDTA (liquid)	2/6	6-17 (5.5)	4-5 (1.5)	158‡ (26)
Jelco†	None	0/5	ND	ND	ND
	Heparin (solid)	0/6	ND	ND	ND
Becton-Dickinson†	None	0/3	ND	ND	ND
	Heparin (solid)	0/2	ND	ND	ND
	EDTA (liquid)	0/3	ND	ND	ND
	Na citrate (liquid)	0/2	ND	ND	ND

NDMA = *N*-Nitrosodimethylamine NDEA = *N*-Nitrosodiethylamine
 NMOR = *N*-Nitrosomorpholine ND = None detected

*Ranges include positive samples only; values in brackets are the means.

†See footnote on p. 31.

‡Only one of the six samples was positive.

Table 2. Nitrosamines in commercial rubber stoppers from evacuated blood-collection tubes

Manufacturer	Additive	Positive stoppers (no./no. tubes tested)	Nitrosamine levels (ppb)*		
			NDMA	NDEA	NMOR
Kimble-Terumot	None	5/6	11-35 (21)	8-25 (16)	85-483 (266)
	Na citrate (liquid)	8/8	14-98 (66)	14-67 (41)	102-678 (420)
	EDTA (liquid)	4/6	20-91 (40)	15-66 (27)	144-467 (320)
Jelco†	None	7/7	39-117 (68)	42-92 (53)	359-1302 (770)
	Heparin (solid)	7/7	40-147 (69)	22-72 (41)	372-770 (514)
Becton-Dickinson†	None	0/3	ND	ND	ND
	Heparin (solid)	0/4	ND	ND	ND
	EDTA (liquid)	0/3	ND	ND	ND
	Na citrate (liquid)	0/4	ND	ND	ND
	K oxalate (solid)	0/3	ND	ND	ND
	Na fluoride (solid)	0/4	ND	ND	ND
	Serum-integrated (solid)	0/3	ND	ND	ND

NDMA = N-Nitrosodimethylamine NDEA = N-Nitrosodiethylamine
 NMOR = N-Nitrosomorpholine ND = None detected

* Ranges include positive samples only; values in brackets are the means.

† See footnote on p. 31.

ton-Dickinson or Corning stoppers contained detectable concentrations of nitrosamines.

GLC-high resolution MS confirmed the presence of NDMA, NDEA and NMOR in the tested rubber stoppers. Analyses were carried out on selected lots of specimen tubes that indicated the possibility that they may contain large quantities of nitrosamines.

Because secondary amines are the most common precursors of nitrosamines, analyses for DMA, DEA and MOR were carried out. The amine and corresponding nitrosamine concentrations in a number of stoppers and collection-tube contents are shown in Table 3. Stoppers in the Kimble-Terumo and Jelco products contained substantial concentrations of the respective amines, ranging up to 1439 ppm MOR. The

solution of sodium citrate in the Kimble-Terumo tube had low levels of DMA and DEA, but 99 ppm MOR. The liquid EDTA anticoagulant had 23 ppm MOR and less than 1 ppm of the other amines. Not more than 2 ppm of the amines was present in all other tubes analysed. Rubbers stoppers in which appreciable levels of amines were found invariably also contained nitrosamines.

Rubber septa used in GLC injection ports were examined to determine whether they too might contain nitrosamines, even though the possibility of sample contamination from this source seemed slight. Precut septa and rubber sheeting used to make septa were extracted for nitrosamines, and of five samples tested, one contained 36 ppb NMOR.

Table 3. Concentration of amines and corresponding nitrosamines in stoppers and tubes

Manufacturer	Additive	Sample	DMA (ppm)	NDMA (ppb)	DEA (ppm)	NDEA (ppb)	MOR (ppm)	NMOR (ppb)	
Kimble-Terumo*	None	Stopper	72	10	69	8	707	85	
		Contents	2	ND	2	ND	3	ND	
	Na citrate (liquid)	Stopper	44	82	82	16	1081	569	
		Contents	4	13	6	7	99	118	
	EDTA (liquid)	Stopper	55	32	65	20	558	251	
		Contents	<1	14	<1	5	23	79	
Jelco*	None	Stopper	81	115	80	78	801	1015	
		Contents	<1	ND	<1	ND	<1	ND	
	Heparin (solid)	Stopper	67	102	78	55	1439	589	
		Contents	<1	ND	<1	ND	2	ND	
	Becton-Dickinson*	None	Stopper	<1	ND	<1	ND	<1	ND
			Contents	<1	ND	<1	ND	<1	ND
EDTA (liquid)		Stopper	<1	ND	<1	ND	<1	ND	
		Contents	<1	ND	<1	ND	<1	ND	
Na citrate (liquid)	Stopper	<1	ND	<1	ND	<1	ND		
	Contents	<1	ND	<1	ND	<1	ND		

DMA = Dimethylamine NDMA = N-Nitrosodimethylamine DEA = Diethylamine NDEA = N-Nitrosodiethylamine
 MOR = Morpholine NMOR = N-Nitrosomorpholine ND = None detected

* See footnote on p. 31.

DISCUSSION

Some amines and nitrosamines themselves are used in the formulation and curing of rubber. Aniline, substituted anilines and diphenylnitrosamines are used as antioxidants, and dimethylamine, *N*-(2,6-dimethylmorpholine)-2-benzothiazole and *p*-nitrosodiphenylamine are used as accelerators in the vulcanization of rubber (Kirk & Othmer, 1953). These nitrogenous compounds may be contaminated with nitrosamines, or they may react with nitrosating agents to form additional nitrosamines.

The data gathered in this study indicate that some, but not all, rubber stoppers obtained from blood-collection tubes contain nitrosamines, and that these compounds are readily leached into the liquids with which they come into contact.

The purpose of this communication is to alert other investigators to the possibility of accidentally introducing nitrosamine contaminants into their studies via products made of rubber. It is essential, therefore, that all blood-collection tubes be surveyed for these contaminants prior to use in studies on nitrosamines.

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