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Note

Rapid method for quantitative analysis of N,N-dibutylnitrosamine, N-butyl-N-(4-hydroxybutyl)nitrosamine and N-butyl-N-(3-carboxypropyl)-nitrosamine in rat urine by gas chromatography—thermal energy analysis

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In 1964 Druckrey et al. [1] reported that among 63 N-nitroso compounds tested, only N,N-dibutylnitrosamine (DBNA) and its ω-hydroxylated derivative, N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), were carcinogenic to the rat urinary bladder. Since then, the metabolism of these compounds has been elucidated and their urinary metabolites have been identified [2]. One of the DBNA metabolic pathways leads to the formation of BBN which is further oxidized to the corresponding acidic derivative N-butyl-N-(3-carboxypropyl)-nitrosamine (BCPN). BCPN has been shown to be the proximate metabolite of both DBNA and BBN responsible for the induction of urinary bladder tumors in rats [2].

DBNA has been observed as a pollutant in tobacco smoke, corrosion inhibitors, food and more recently in several rubber products including baby bottle nipples, toys, and possibly new motor cars [3—8]. Because of the widespread distribution of DBNA in the environment, a specific, sensitive analytical method for evaluating human exposure to this N-nitroso compound is very much needed. This paper describes the development of a gas chromatographic (GC) method for the quantitative analysis of DBNA, BBN and BCPN extracted from urine of rats given DBNA. BBN was measured as its trimethylsilyl ether (BBN-TMS) and BCPN as its trimethylsilyl ester (BCPN-TMS). The high sensitivity required was achieved by using a gas chromatograph coupled with a thermal energy analyzer (GC—TEA).

The application of the method to human urine samples will be discussed elsewhere.

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EXPERIMENTAL

Reagents

N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and pyridine were obtained from Pierce Chemical Co., Rockford, IL, U.S.A.; Clin Elut™ 1020 extraction columns were produced by Analytichem International, Harbor City, CA, U.S.A.; β-glucuronidase/arylsulfatase was obtained from Boehringer (Mannheim, G.F.R.).

Standards

DBNA was purchased from Eastman Kodak Co., Rochester, NY, U.S.A.; BBN and BCPN were kindly donated by Dr. Masashi Okada of the Tokyo Biomedical Research Institute, Japan.

Urine extraction

Male CD-COBS rats (body weight 180 ± 10 g) obtained from Charles River, Italy, were fasted overnight before treatment and housed individually in metabolic cages. DBNA was administered orally at the dose of 5 mg/kg. Urine samples were collected for 24 h on 500 mg of ammonium sulfamate, added to inhibit unwanted nitrosation. Each 24-h urine sample was divided into two and the volume of each half was made up to 20 ml with water if less than that, and the pH was adjusted to 5. Half the samples were incubated with β-glucoronidase/arylsulfatase (50 μl/sample) at 37°C overnight. All samples were extracted twice with 10 ml of ethyl acetate. The organic phases were combined (18 ml) and added to Clin Elut columns to which 20 ml of 0.1 *N* hydrochloric acid had been added and absorbed. After 2–5 min a second 18 ml of ethyl acetate was added to the columns; the column eluate was collected and the volume was reduced to 0.2 ml; 1—4 μl were analyzed by GC—TEA for DBNA content.

The samples were then evaporated to dryness. The dry residue was dissolved in 50 μl of ethyl acetate and 50 μl of reagent mixture containing pyridine—BSTFA—TMCS (50:45:5); 1—4 μl were analyzed by GC—TEA for BBN and BCPN content. In order to confirm the identity of the gas chromatographic peaks, GC—mass spectrometry (MS) analyses were carried out.

Gas chromatography and gas chromatography—mass spectrometry

A DANI 3800 gas chromatograph equipped with a TEA 543 detector (Thermal Energy Analyzer, Thermo Electron) was used. The glass column (2 m × 2 mm I.D.) was packed with 3% OV-1 on Gas-Chrom Q, 100—120 mesh. DBNA was analyzed at 140°C. For analysis of BBN and BCPN the oven temperature was kept at 140°C for 2 min, then programmed from 140 to 170°C at a rate of 15°C/min. The carrier gas (helium) flow-rate was 30 ml/min. The GC—TEA interface temperature was 250°C and the pyrolyzer temperature was 500°C.

GC—MS analysis was performed on an LKB 2091-051 gas chromatograph—mass spectrometer equipped with an LKB 2130 computer system for data acquisition and calculation and used in the electron impact mode. GC—MS operating conditions were as follows: glass WCOT open tubular column 25 m × 0.3 mm I.D. coated with OV-1, film thickness 0.15 μm; column head

pressure, 2 bars. DBNA analysis was performed at 120°C; for the analysis of BBN and BCPN the temperature was kept at 120°C for 2 min then programmed from 120 to 200°C at a rate of 4°C/min; ion source temperature, 250°C; electron energy, 70 eV; trap current, 50 μA; accelerating voltage, 3.5 kV; resolution 600; scan speed 3.

RESULTS AND DISCUSSION

In the GC—TEA condition described, DBNA had a retention time of 2 min 30 sec; BBN-TMS and BCPN-TMS had retention times of 5 and 6 min, respectively. A linear response was observed for all three compounds for injected amounts ranging from 0.5 to 4 ng, the correlation coefficient r being 0.999, 0.9993 and 0.9992 for DBNA, BBN, and BCPN, respectively.

Extraction and clean-up efficiency was evaluated by adding known amounts (25—200 ng) of standard DBNA, BBN, and BCPN to urine samples (20 ml) from untreated rats. Recovery values for DBNA, BBN and BCPN were 70 ± 2%, 67 ± 4% and 72.5 ± 2% (mean ± S.D.), respectively. The lowest detectable amount of the three compounds was 10 ng excreted in 24 h.

Fig. 1 shows a typical GC—TEA chromatogram of a blank urine sample (Fig. 1a), a urine sample spiked with 50 ng of DBNA (Fig. 1b) and a urine sample from DBNA-treated animals (Fig. 1c).

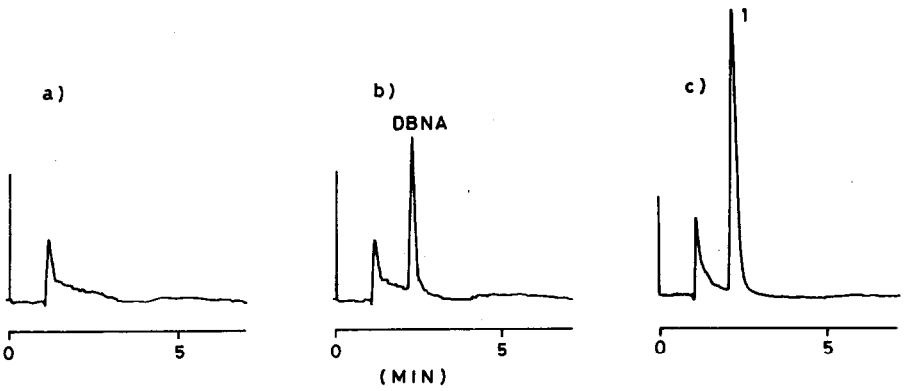

Fig. 1. GC—TEA chromatograms of a blank urine sample (a), a urine sample spiked with 50 ng of DBNA (b), and a urine sample from DBNA-treated animals (c). Peak 1 had the same retention time as standard DBNA.

Fig. 2 shows a typical GC—TEA chromatogram of a blank sample of urine after derivatization with BSTFA—TMCS (Fig. 2a) and a urine sample spiked with BBN and BCPN (50 ng each) and analyzed after derivatization with BSTFA—TMCS (Fig. 2b). Fig. 3 shows a typical GC—TEA chromatogram of a urine sample from DBNA-treated animals analyzed for BBN and BCPN content before (Fig. 3a) and after (Fig. 3b) hydrolysis with β-glucuronidase/aryl-sulfatase. Samples were derivatized with BSTFA—TMCS before analysis.

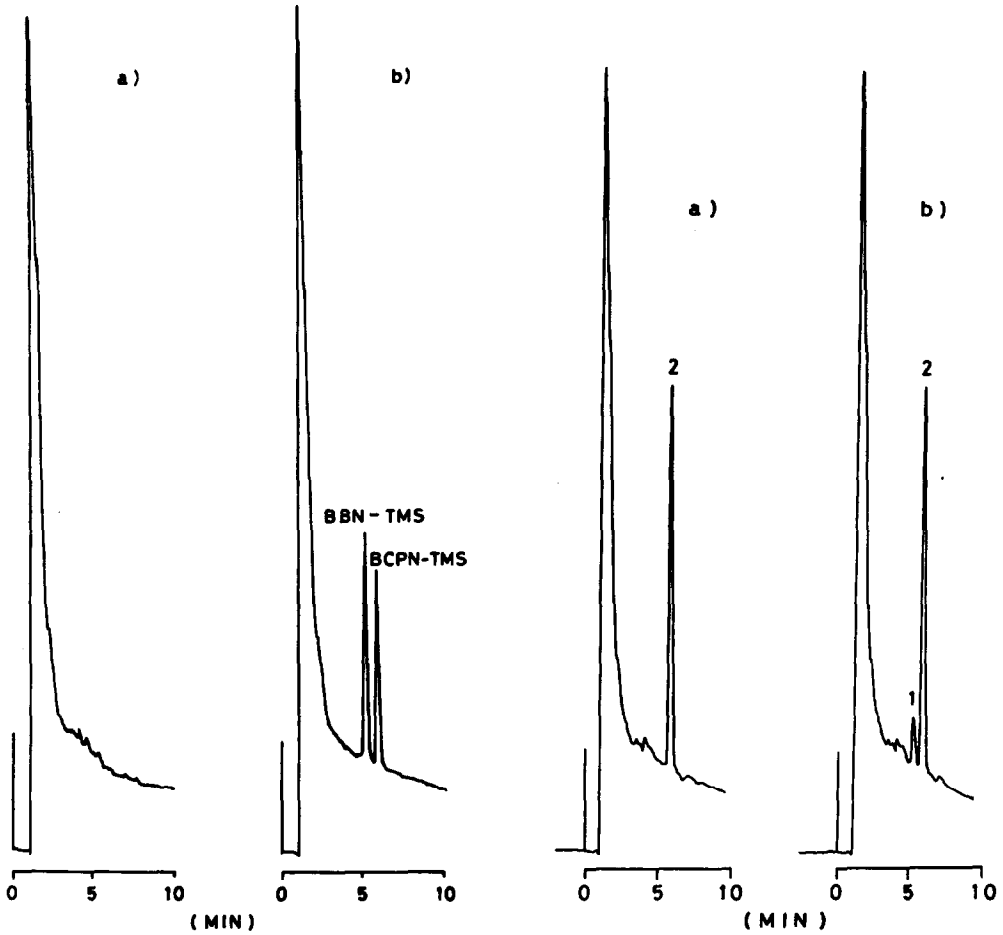

Fig. 2. GC—TEA chromatograms of a blank urine sample after derivatization with BSTFA—TMCS (a) and a urine sample spiked with BBN and BCPN (50 ng each) and analyzed after derivatization with BSTFA—TMCS (b).

Fig. 3 GC—TEA chromatograms of a urine sample from DBNA-treated animals analyzed for BBN and BCPN content before (a) and after (b) hydrolysis with β-glucuronidase/arylsulfatase. Samples were derivatized with BSTFA—TMCS. Peaks 1 and 2 had the same retention time as standard BBN-TMS and BCPN-TMS, respectively.

Urinary levels of DBNA, BBN and BCPN in rats given DBNA at the oral dose of 5 mg/kg are reported in Table I. About 0.3% of the administered DBNA is excreted unchanged in the 24-h urine; BBN is present only in the conjugated form and amounts to 0.05% of the administered DBNA. BCPN is about 21% of the administered DBNA and is present as free acid. Quantitatively, these results differ slightly from those published in an earlier paper [9], whose purpose, however, was to identify DBNA urinary metabolites rather than to quantify them.

TABLE I

DBNA, BBN AND BCPN IN URINE OF RATS GIVEN DBNA 5 mg/kg PER OS

Compound	μg per 24 h (mean ± S.E.)	
	Free	Glucuronic acid/aryl-sulfate conjugated
DBNA	2.7 ± 0.75	—
BBN	n.d.*	0.46 ± 0.13
BCPN	191.1 ± 21	n.d.

*n.d. = not detectable (< 10 ng per 24 h).

The identity of the GC—TEA peaks of DBNA, BBN and BCPN extracted from urine of animals treated with DBNA was verified by GC—MS. Because of the small amount of BBN excreted in the urine, several samples had to be pooled to give enough material for a mass spectrum. The three mass spectra were exactly the same as those obtained by analyzing authentic standards.

The DBNA fragmentation pattern was in agreement with the data reported in the literature [10]. In the mass spectra of BBN-TMS and BCPN-TMS, the molcular ions at *m/z* 246 and 260, as well as in the mass spectra of most silylated compounds, are never present, but the $M^+ - CH_3$ peaks (*m/z* 231 and 245, respectively) can be used for determination of molecular weight [11]. The fragment assignments for BBN-TMS and BCPN-TMS mass spectra are reported in Table II.

TABLE II

MASS SPECTRAL DATA OF BBN AND BCPN AS THEIR TMS DERIVATIVES

BBN		BCPN		Fragment
m/z	%	*m/z*	%	
246	0	260	0	$[M]^+$
231	6	245	6	$[M - CH_3]^+$
229	11			$[M - OH]^+$
216	5	230	34	$[M - NO]^+$
172	7	186	7	$[M - (C_3H_7+HNO)]^+$
158	12	172	21	$[M - (NO+CH_3+C_3H_7)]^+$
		159	18	$[M - (C_3H_7+HNO+HCN)]^+$
126	100	140	29	$[M - (NO+TMS\text{-}OH)]^+$
		112	47	$[M - (COOTMS+HNO)]^+$
		98	31	$[M - (NO+TMS\text{-}OH+C_3H_6)]^+$
84	47	84	62	$CH_3\text{-}CH_2\text{-}CH_2\text{-}CH{=}\overset{+}{N}{=}CH_2$ or $CH_3\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}\overset{+}{N}{\equiv}CH$

Because of its high sensitivity and specificity, the method described appears to be useful for assessing DBNA contamination by measuring the amount of the nitrosamine and its oxidized metabolites BBN and BCPN in urine. The amount of urinary BCPN is the better indicator of the degree of contamination

since this metabolite is excreted in higher concentrations than BBN or DBNA itself; moreover, it is the proximate metabolite responsible for the induction of urinary bladder tumors in rats.

The method will be used to check the urine of human populations likely to be exposed to DBNA, e.g. rubber industry workers, or bottle-fed babies.

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REFERENCES

1 H. Druckrey, R. Preussmann, S. Ivankovic, D. Schmähl, J. Afkham, G. Blum, H.D. Mennel, M. Müller, P. Petropoulos and H. Schneider, Z. Krebsforsch., 69 (1967) 103.
2 M. Okada and M. Ishidate, Xenobiotica, 7 (1977) 11.
3 A. McCormick, M.J. Nicholson, M.A. Baylis and J.G. Underwood, Nature (London), 244 (1973) 237.
4 M.C. Archer and J.S. Wishnok, J. Environ. Sci. Health, A11 (1976) 583.
5 T.A. Gough, IARC Sci. Publ., 19 (1978) 297.
6 C.B. Ireland, F.P. Hytrek and B.A. Lasoski, Amer. Ind. Hyg. Ass. J., 41 (1980) 895.
7 B. Spiegelhalder and R. Preussmann, IARC Sci. Publ., 41 (1982) 231.
8 D.P. Rounbehler, J. Reisch and D.H. Fine, Food Cosmet. Toxicol., 18 (1980) 147.
9 E. Suzuki and M. Okada, Gann, 71 (1980) 863.
10 W.T. Rainey, W.H. Christie and W. Lijinsky, Biomed. Mass Spectrom., 5 (1978) 395.
11 A.E. Pierce, Silylation of Organic Compounds, Pierce Chemical Company, Rockford, IL, 1968, p. 33.