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DETERMINATION OF N-MONONITROSOPIPERAZINE AND N,N'-DINITROSOPIPERAZINE IN HUMAN URINE, GASTRIC JUICE AND BLOOD

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SUMMARY

A rapid, selective and sensitive method for the determination of N-mononitrosopiperazine and N,N'-dinitrosopiperazine in human urine, gastric juice and blood has been developed using gas—liquid chromatography with thermal energy analysis. The compounds were isolated by extraction and Extrelut® with dichloromethane or chloroform. The detection limits were between 0.2 and 2 ng/ml.

INTRODUCTION

Piperazine is a widely used anthelmintic drug. It is rapidly nitrosated under in vitro conditions in gastric juice and human saliva to yield N-mononitrosopiperazine (MNPZ) and N,N'-dinitrosopiperazine (DNPZ) [1—5]. In vivo nitrosation of ingested piperazine with nitrite has also been reported [5—7]. Recently we found MNPZ in human gastric juice and urine after ingestion of piperazine alone [8]. DNPZ, and possibly MNPZ, are carcinogenic to laboratory animals [9—11].

Several methods have been developed for the determination of MNPZ and DNPZ in biological fluids. Some of these methods, based on spectrophotometric [4, 12] and thin-layer chromatographic [3] procedures, are only semi-quantitative with a low sensitivity. Gas—liquid chromatographic

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(GLC) methods, using nitrogen-specific flame ionisation detection [6, 7], have a higher sensitivity and selectivity. Recently, a high-performance liquid chromatographic method has been reported [5].

However, these methods are not sufficiently sensitive or specific for analysis of low levels of MNPZ and DNPZ. This report describes a rapid, selective and sensitive method for the determination of MNPZ and DNPZ in human urine, gastric juice and blood, using a gas—liquid chromatograph interfaced with a thermal energy analyzer (TEA) detector. The TEA detector exhibits extreme sensitivity and high specificity to N-nitrosamines [13].

EXPERIMENTAL

Reagents

Dichloromethane, chloroform, 2,2,4-trimethylpentane and methanol (all analytical grade) were obtained from E. Merck (Darmstadt, F.R.G.) and were used without further purification. Water was deionized. Kieselguhr (Extrelut®, Merck) was dried overnight at 200°C prior to use and stored at the same temperature; prepacked Extrelut columns were also used. Piperazine syrup (48 mg/ml) was obtained from ACO Läkemedel (Solna, Sweden). All other chemicals were reagent grade or better. All the reagents, organic solvents and deionized water were checked to ensure absence of interfering substances. Trace amounts of MNPZ (3—20 μg/g piperazine) were found in the piperazine syrup.

Standards

A stock solution of MNPZ (100 μg/ml) in 2,2,4-trimethylpentane was obtained from Thermo Electron (Waltham, MA, U.S.A.). A stock solution of DNPZ (100 μg/ml) was prepared by dissolving DNPZ in 2-propanol.

For GLC calibration, working standard solutions (400 ng/ml) of MNPZ in methanol—water (95:5, v/v), and of DNPZ in methanol were prepared. For recovery experiments standard solutions of MNPZ in water or methanol—water (95:5, v/v), and of DNPZ in methanol were prepared.

Stock solutions of MNPZ and of DNPZ were stored at about −15°C, and standard solutions and working solutions of MNPZ and DNPZ at about 4°C.

Apparatus

A Varian 2700 (Palo Alto, CA, U.S.A.) gas chromatograph interfaced with a thermal energy analyzer (TEA, Model 502, Thermo Electron) was used. The GLC furnace was removed from the TEA and connected to the GLC column via a 5.5-cm long glass tube. The glass column (1.8 m × 1.9 mm I.D.) was packed with 15% SE-52 on Chromosorb W HP.

Chromatographic conditions

The operating conditions for GLC—TEA were as follows: column temperature 190°C, injector temperature 240°C, helium flow-rate about 38 ml/min, furnace temperature 475°C, oxygen flow-rate about 5 ml/min, vacuum pressure about 1 mmHg, cold trap temperature −150 to −160°C.

Extraction procedures

Urine. One millilitre of 2 *M* sodium hydroxide was added to 20 ml of urine. If the sample was not worked up at once, 1 ml of sodium sulphamate solution (4 g of sulphamic acid and 3 g of sodium hydroxide per 100 ml of water) was added as a nitrite trap, and 2 ml of 2 *M* sodium hydroxide were added prior to extraction. The mixture was poured on to an Extrelut column (23 cm × 2 cm I.D.), and after 10—15 min the nitrosamines were eluted with 100 ml of dichloromethane. The eluate was evaporated to dryness, and the residue was dissolved in 0.5—1 ml of methanol—water (95:5, v/v). The volume was measured with a 1000-μl Hamilton syringe, and 5-μl aliquots were injected into the gas chromatograph.

Gastric juice. One millilitre of 2 *M* sodium hydroxide was added to 10 ml of gastric juice. The sample was immediately put on a prepacked Extrelut column or a 23-cm long Extrelut column followed by 10 ml of water and analyzed as described above for urine.

Blood. Five millilitres of the blood sample were treated with 15 ml of 0.025% ammonium hydroxide for 10—15 min. The mixture was added to an Extrelut column and the nitrosamines were eluted with 100 ml of chloroform. The eluate was concentrated and analyzed as outlined above.

Recovery studies

To measure recovery, various concentrations of MNPZ and/or DNPZ were added to each body fluid studied, and the samples were extracted as described above. The recovery was determined by comparing peak areas of MNPZ and DNPZ in the sample with peak areas obtained by direct injection of standard solutions.

Calculations

The nitrosamine concentrations were always calculated by the external standard method, using peak area or peak height ratios. A new standard calibration was performed every day.

Artifact formation

Gastric juice. To 10 ml of gastric juice were added, in the following order, 2 ml of 2 *M* sodium hydroxide, 0.5 g of sulphamic acid or ascorbic acid, 1 ml of diluted piperazine syrup (10 mg/ml) and, finally, 1 ml of sodium nitrite (5 mg/ml). In one experiment, sodium hydroxide was added last instead of first to give a pH 12—14. Samples without sulphamic or ascorbic acid were also analyzed for artifact formation. The samples were extracted immediately or after five days' storage at about 5°C, and analyzed as described above.

Urine. To 10 ml of urine containing piperazine (3 mg) and nitrite (22 μg) were added 0.1—2.0 ml of 4% sulphamic acid, 0.1—2.0 ml of sodium sulphamate (4 g of sulphamic acid and 3 g of sodium hydroxide per 100 ml of water), or 200—2000 mg of urea. These samples, and samples without nitrosation inhibitors, were extracted after storing for one day at 5°C and analyzed as outlined above.

RESULTS AND DISCUSSION

Extraction

The use of Extrelut for extraction gave better results than those obtained with earlier described extraction methods [2—7]. Fig. 1 shows a chromatogram of an extract of a gastric juice sample spiked with 28.8 ng/ml MNPZ and 4.8 ng/ml DNPZ. The overall analytical recovery for DNPZ (8—32 ng/ml) was in the range of 80—96% in all media (Table I), while for MNPZ (8—32 ng/ml) the recovery varied between 51 and 78%. The coefficients of variation within the range of levels tested varied from 3.6 to 23.7% for MNPZ and from 0.97 to 8.7% for DNPZ. The poor recovery of MNPZ was probably due to adsorption losses on the Extrelut column.

Artifact formation

When performing analysis of N-nitroso compounds in the ppb range, it is necessary to prevent artifact formation during the analysis [14]. Earlier studies [3, 12, 15] have shown that ascorbic acid, glutathione, ammonium

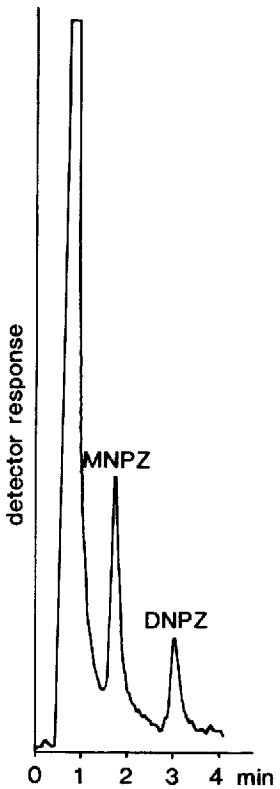

Fig. 1. Chromatogram of an extract of gastric juice spiked with 28.8 ng/ml MNPZ and 4.8 ng/ml DNPZ.

TABLE I

RECOVERY OF MNPZ AND DNPZ FROM URINE, GASTRIC JUICE AND BLOOD

Body fluid	Amount added (ng/ml)	No. of determinations		Recovery (%, mean ± S.D.)		Coefficient of variation (%)	
		MNPZ	DNPZ	MNPZ	DNPZ	MNPZ	DNPZ
Urine	8	4	4	63 ± 6.1	94 ± 6.8	9.7	7.2
	16	4	4	74 ± 2.7	93 ± 1.9	3.6	2.0
	32	4	4	75 ± 5.8	96 ± 4.6	7.8	4.8
Gastric juice	8	4	4	66 ± 5.4	92 ± 4.0	8.2	4.4
	16	4	4	73 ± 10.9	84 ± 5.2	15.0	6.2
	32	4	4	78 ± 3.2	80 ± 5.7	4.2	7.1
Blood	8	4	4	52 ± 12.2	88 ± 7.7	23.7	8.7
	16	4	4	51 ± 8.8	85 ± 3.6	17.1	4.2
	32	4	4	63 ± 3.9	86 ± 0.83	6.1	0.97

TABLE II

ARTIFACT FORMATION OF MNPZ AND DNPZ IN ANALYSIS OF GASTRIC JUICE

To 10 ml of gastric juice were added 2 ml of 2 *M* sodium hydroxide, 0.5 g of sulphamic or ascorbic acid (as nitrosation inhibitor), 1 ml of diluted piperazine syrup (10 mg/ml), and 1 ml of sodium nitrite (5 mg/ml). The samples were extracted immediately or after five days, and analyzed as described in the Experimental section. ND = not detected (< 0.005 μg); tr = trace (0.005—0.015 μg).

Method	Nitrite inhibitor	Time before extraction (days)	Nitrosamine detected* (μg)	
			MNPZ	DNPZ
Sodium hydroxide added first	None	0	tr	ND
		5	tr	ND
	Sulphamic acid	0	tr	0.023
		5	tr	0.046
	Ascorbic acid	0	tr	ND
		5	tr	ND
Sodium hydroxide added last	None	0	3590	715
	Sulphamic acid	0	1.4	ND
	Ascorbic acid	0	1.3	ND

*Not corrected for recovery.

sulphamate and urea inhibit the formation of MNPZ. However, the inhibition is sometimes only partial, depending on the pH. In the analysis of gastric juice, the samples must immediately be made alkaline to block artifact formation (Table II). The presence of sulphamic acid resulted in the formation of measurable amounts of DNPZ in the analysis of gastric juice spiked with piperazine and nitrite. Earlier, ascorbic acid has been reported to increase

TABLE III

ARTIFACT FORMATION OF MNPZ AND DNPZ IN ANALYSIS OF URINE

To 10 ml of urine containing piperazine (3 mg) and sodium nitrite (22 μg) were added sulphamic acid, sodium sulphamate or urea (as nitrosation inhibitor). The samples were extracted and analyzed after one day as described in the Experimental part. ND = not detected (< 0.005 μg); tr = trace (0.005—0.015 μg).

Nitrosation inhibitor	Amount of inhibitor added		Nitrosamine detected* (μg)	
	ml	g	MNPZ	DNPZ
Sulphamic acid (4%)	0	—	tr	ND
	0.1	—	0.020	ND
	0.2	—	0.025	ND
	0.5	—	0.114	ND
	1.0	—	0.273	ND
	2.0	—	0.017	ND
Sodium sulphamate (4%)	0	—	tr	ND
	0.1	—	tr	ND
	0.2	—	tr	ND
	0.5	—	tr	ND
	1.0	—	ND	ND
	2.0	—	tr	ND
Urea	—	0	0.073	ND
	—	0.4	0.065	ND
	—	2.0	0.051	ND

*Not corrected for recovery.

the formation of DNPZ [3]. In the analysis of urine, the nitrosation was effectively blocked by sodium sulphamate (Table III), while sulphamic acid increased the formation of MNPZ. The reason for this is probably the pH change on the addition of sulphamic acid.

In the analysis of blood, an unidentified NO-positive peak occasionally appeared in the chromatogram (Fig. 2). An enhancement of this signal always reduced the MNPZ signal and the recovery of MNPZ. With a high unidentified peak, the recovery of MNPZ was decreased to 6—7%. The DNPZ level was not affected by this unknown peak.

Standards

It has earlier been reported that DNPZ may be unstable if stored in dichloromethane for more than two to three days [3]. We found no decomposition of DNPZ in the solvents used: 2,2,4-trimethylpentane, 2-propanol, dichloromethane, chloroform, methanol, and methanol—water (95:5, v/v). On the other hand, MNPZ was unstable in methanol, but stable in methanol—water (95:5, v/v), water, 2,2,4-trimethylpentane, dichloromethane, and chloroform. In methanol an additional peak with a slightly longer retention time than MNPZ could be observed after storage.

Standard solutions of MNPZ and DNPZ contained two unidentified NO-

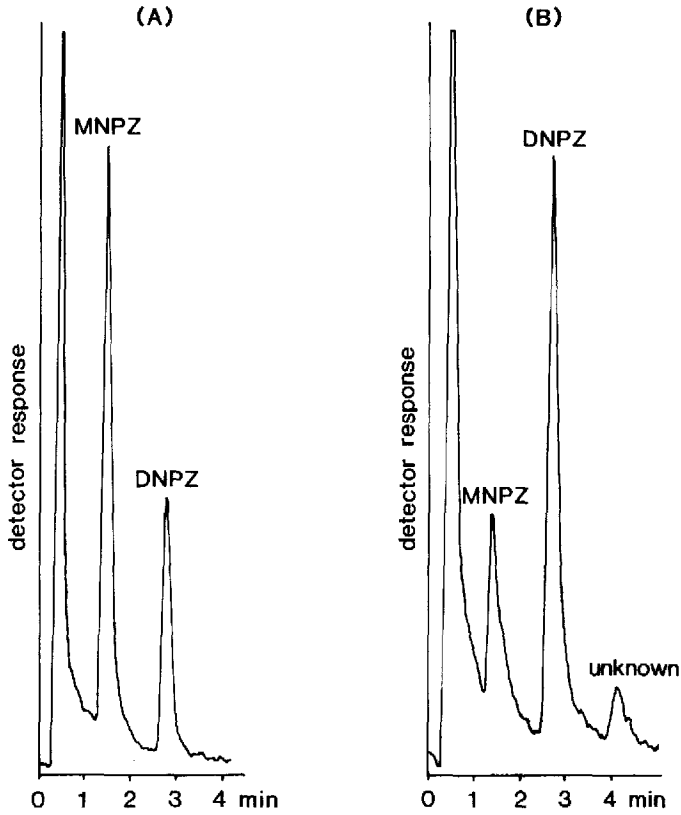

Fig. 2. Chromatograms of extract of blood: (A) spiked with 108 ng/ml MNPZ and 18.0 ng/ml DNPZ; and (B) spiked with 40.0 ng/ml MNPZ and 46.7 ng/ml DNPZ.

positive peaks with retention times about 5 sec longer than MNPZ and DNPZ. These unknown impurities are, in contrast to MNPZ and DNPZ, stable upon ultraviolet radiation.

Detection

In our GLC—TEA system the furnace has been removed from the TEA instrument and connected directly to the GLC column. The short distance between the end of the column and the furnace contributes to the high sensitivity in the analysis of MNPZ and DNPZ.

For GLC calibration and determination of MNPZ, we found that methanol—water (95:5, v/v) was the most suitable solvent. The use of 2,2,4-trimethylpentane gave poor reproducibility at low concentrations, due to partial loss of MNPZ in the syringe.

Good linearity was obtained in the relationship between peak areas and concentrations of MNPZ and DNPZ in standard solutions in the range 5.0—400 ng/ml (Fig. 3). The coefficient of variation was 4.8% for MNPZ (n = 10) and 4.6% for DNPZ (n = 10) at a concentration of 400 ng/ml. To obtain a high sensitivity and a good precision in the GLC calibration and determination, the GLC column was activated by repeated injections of blank

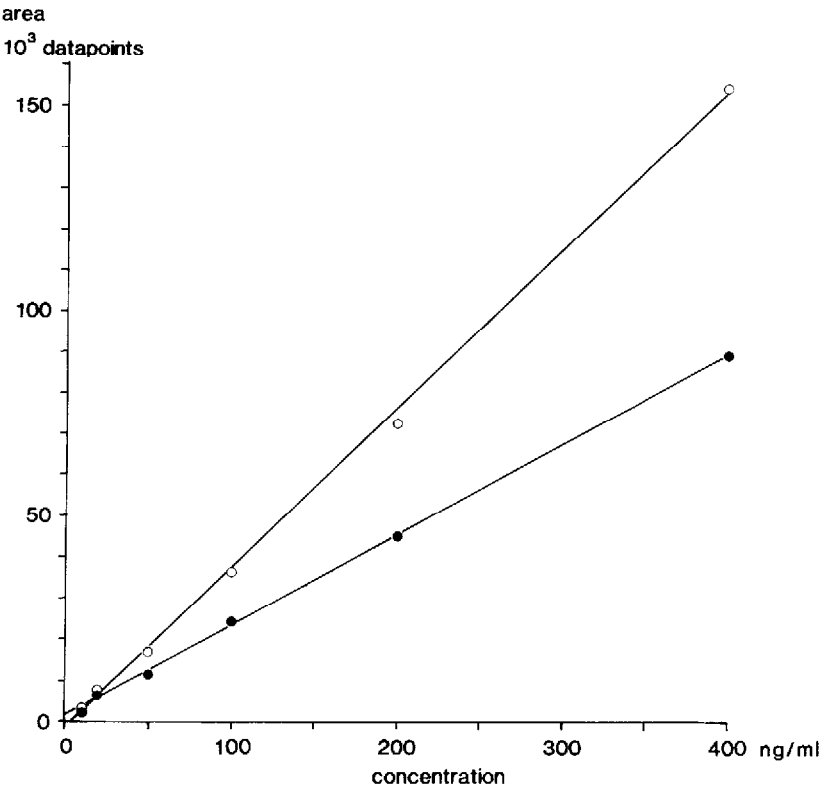

Fig. 3. Standard curves of MNPZ (○) and DNPZ (●) constructed by plotting the peak areas against known concentrations of MNPZ and DNPZ in the range 5.0—400 ng/ml.

urine samples taken through the extraction procedures. One or two 5-μl injections of methanol—water (95:5, v/v) were sometimes required to eliminate memory effects after injections of large amounts of MNPZ and/or DNPZ.

The minimum detectable concentrations of MNPZ and DNPZ in urine were 0.2 and 0.3 ng/ml, in gastric juice 0.4 and 0.5 ng/ml, and in blood 2.0 and 2.0 ng/ml. The signal-to-noise ratio was at least 3:1 for both MNPZ and DNPZ at these concentrations.

Application

The technique was applied to study the in vivo nitrosation of piperazine in the stomach of man [8, 16]. Piperazine syrup was given to fasting volunteers. Urine, gastric juice and blood samples were taken at different times relative to the intake of piperazine. Fig. 4. shows a chromatogram of an extract of a urine sample containing 3.7 ng/ml MNPZ from a volunteer 4 h after the intake of 0.51 g of piperazine and 2.0 g of ascorbic acid. Routine recoveries of MNPZ and DNPZ from urine, gastric juice, and blood in the in vivo nitrosation study, covering a period of about one year, are shown in Table IV. The gastric juice samples were worked up immediately after sampling, and then mailed from Lund to Uppsala for analysis (24 h). The urine (containing sodium sulphamate solution) and blood samples were sent to Uppsala, where the samples were extracted and analyzed.

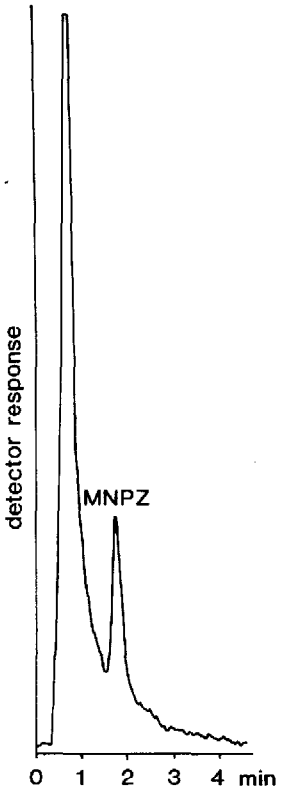

Fig. 4. Chromatogram of an extract of 20 ml of urine containing 3.7 ng/ml MNPZ from a volunteer 4 h after intake of 0.51 g of piperazine and 2.0 g of ascorbic acid.

TABLE IV

EXTRACTION YIELD OF MNPZ AND DNPZ FROM AN APPLICATION OF THE METHOD COVERING A PERIOD OF ABOUT ONE YEAR

Medium	Concentration (ng/ml)	No. of determinations		Recovery (%, mean ± S.D.)	
		MNPZ	DNPZ	MNPZ	DNPZ
Urine	2— 14	17	16	84 ± 12	90 ± 12
	20— 140	22	14	84 ± 16	102 ± 12
	200—1400	4	4	84 ± 8	99 ± 10
Gastric juice	2— 14	4	12	72 ± 17	89 ± 9
	20— 140	6	9	70 ± 16	95 ± 16
	200—1400	7	3	90 ± 10	101 ± 9
Blood	2— 14	10	6	24 ± 8	42 ± 9
	20— 140	11	16	47 ± 12	100 ± 18

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