Journal of Chromatography, 164 (1979) 95—99
Biomedical Applications

CHROMBIO. 360

Note

Micromethod for the gas chromatographic determination of morpholine in biological tissues and fluids

E.G. TOMBROPOULOS

National Institute of Environmental Health Sciences, National Institutes of Health, P.O. Box 12233, Research Triangle Park, N.C. 27709 (U.S.A.)

(First received September 5th, 1978; revised manuscript received April 24th, 1979)

Morpholine, tetrahydro-2H-1,4-oxazine, is widely used as a corrosion inhibitor, a neutralizer, and a scrubbing agent. Derivatives of morpholine are also used in plasticizers, synthetic lubricants, emulsifiers, and antioxidants. These are also used by pharmaceutical industries. Morpholine, under physiological conditions, can form N-nitrosomorpholine [1,2], a potent carcinogen [3—6]. Morpholine reacts readily even with the low levels of nitrite in saliva [2], to form N-nitrosomorpholine. Because morpholine can readily form N-nitrosomorpholine, it may present a potential hazard to those handling it in large quantities.

In order to evaluate the potential hazard and to study the metabolism of morpholine, a method had to be devised to isolate and determine morpholine levels in tissue and biological fluids. There is no known reference to this procedure in the literature. This paper, therefore, presents a chromatographic method by which microgram levels of morpholine in blood, urine, and biological tissue can be accurately measured.

EXPERIMENTAL

Reagents

Morpholine was obtained from Fisher Scientific (Fairlawn, N.J., U.S.A.). An aqueous stock morpholine solution of 1 mg/ml was used to prepare plasma concentrations of 5, 10, 20, 50, 100, 150, and 400 μg morpholine per ml of plasma. A second aqueous stock solution of 10 mg/ml morpholine was used for injecting liver or lung tissue and for adding to urine samples to achieve concentrations ranging from 25 to 200 μg of morpholine per g of tissue or 0.125 to 2 mg of morpholine per ml of urine. The above blood, urine and tissue concentrations of morpholine were used for recovery studies.

Picric acid, 2,4,6-trinitrophenol, was purchased from Fisher Scientific. A 0.5 *M* solution of picric acid in methanol was used for acidification of samples. All other chemicals used were of reagent grade.

Procedures

Five volumes of methanol were added to a 0.5—5 ml sample of plasma or urine to precipitate the protein and extract the morpholine. The glass stoppered tubes containing the samples in methanol were centrifuged and the supernatant transferred to a round-bottom flask. Tissues were homogenized in a glass homogenizer with 10 volumes of methanol and extracted five times with 10 volumes of methanol (v/w). These extracts were combined and transferred to a round-bottom flask. Extracts from blood, urine and tissues were thereafter processed in a similar manner. These extracts were acidified with picric acid to pH 2—2.5, as determined with pH indicator paper, and then evaporated under vacuum in a rotary evaporator. To the dry residue was added a mixture of diethyl ether and 3 *N* sulfuric acid (4:1) and this was transferred to a centrifuge tube. The amount of sulfuric acid in the mixture of diethyl ether, on a molar basis, was three times the amount of picric acid added previously for acidification. The diethyl ether phase containing the picric acid was discarded. The aqueous phase containing the morpholine was re-extracted twice with diethyl ether to eliminate any picric acid remaining from the first extraction.

The solution containing the morpholine was neutralized with calcium carbonate. The precipitate was washed twice with water. The original supernatant and the washings were combined. Five microliters of the combined extracts were used for injection in the gas chromatograph.

Gas chromatography

A Tracor gas chromatograph, series MT200, equipped with a flame ionization detector, was used in all experiments. A glass column (1.83 m × 4 mm) packed with Chromosorb 103 (100—120 mesh) (Johns-Manville, Denver, Colo., U.S.A.) was found to be most suitable. The glass column was silylated before being packed by filling it with a solution of 5% dichlorodimethylsilane (DCDMS) in toluene. The solution was allowed to stand inside the column for at least 30 min, then the column was rinsed successively with toluene and methanol and dried with a stream of nitrogen.

To pack the column, one end of it was connected to the household vacuum and the other end was connected to a funnel containing the packing material. During the packing, the column was gently tapped. Before the column was used, it was preconditioned at 230° overnight. The following gas chromatographic parameters were used: column temperature, 145° isothermal; detector temperature, 240°; injector temperature, 210°; helium flow-rate at the detector point, 20 ml/min; input attenuator, 1; output attenuator, 1; recorder chart speed, 10 mm/min. The detector had a hydrogen flow-rate of 40 ml/min and an air flow-rate of 325 ml/min. The retention time of morpholine, under these conditions, was 23 min.

RESULTS AND DISCUSSION

Fig. 1A illustrates a chromatogram of blood plasma without morpholine, and Fig. 1B illustrates a chromatogram of blood plasma in which morpholine was added. Fig. 1C is a chromatogram of a urine sample and Fig. 1D is a urine sample spiked with morpholine. The samples containing morpholine and the samples which didn't contain morpholine were treated the same way following the procedure described under Experimental. These figures illustrate the separation of morpholine from other plasma or urine components which could have been co-extracted with morpholine.

Known amounts of morpholine were added to blood samples and their morpholine content was determined following the method described in this paper. A typical standard curve of the above determinations is shown in Fig. 2. The procedure was found to yield linear results from 0 to 200 μg/ml of plasma.

The recovery of morpholine was calculated by comparing the chromatographic peak heights of samples spiked with known amounts of morpholine before being processed, with those of morpholine standards. Differences in concentration of morpholine among the biological fluids and tissues (after inhalation of morpholine vapors) were taken into consideration when the range of morpholine levels was chosen during the recovery studies. The recovery of morpholine from plasma, urine, and liver tissue is shown in Table I.

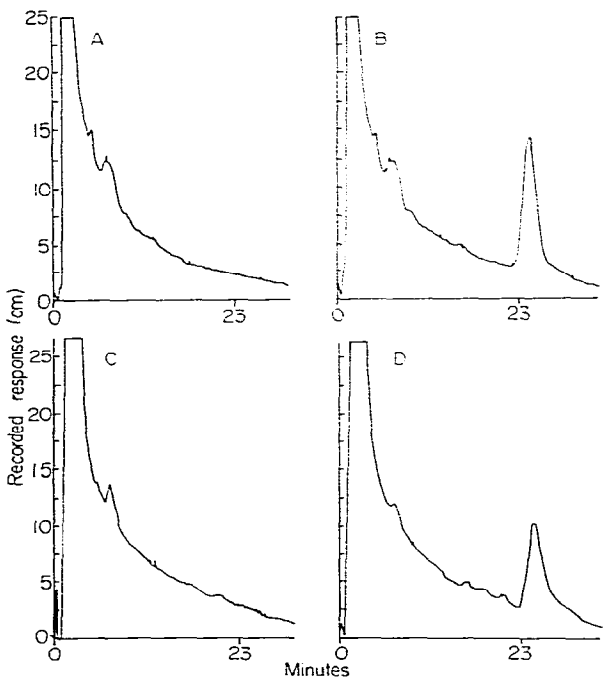

Fig. 1. Gas chromatograms of (A) blood plasma blank; (B) blood plasma with morpholine; (C) urine blank; (D) urine with morpholine. All samples were treated as described in Procedures.

TABLE I

RECOVERY OF ADDED MORPHOLINE FROM PLASMA, URINE AND LIVER TISSUE

Biological fluid or tissue	Amount (μg) of morpholine added per g of tissue or ml of body fluid	Recovery (%)	Standard deviation	Number of replicates
Plasma	5	62.8	3.5	6
	10	70.2	3.6	4
	20	63.5	6.2	6
	50	69.7	7.9	8
	100	63.2	4.2	4
	150	63.0	2.0	4
	400	61.4	2.3	4
Urine	125	55.5	3.5	5
	250	58.1	1.4	4
	500	58.0	1.4	4
	1500	58.5	2.1	4
	2000	57.1	1.6	4
Tissue	25	62.2	4.9	4
	50	67.5	6.4	2
	100	64.5	4.9	2
	150	67.7	5.7	4
	200	60.2	4.5	4

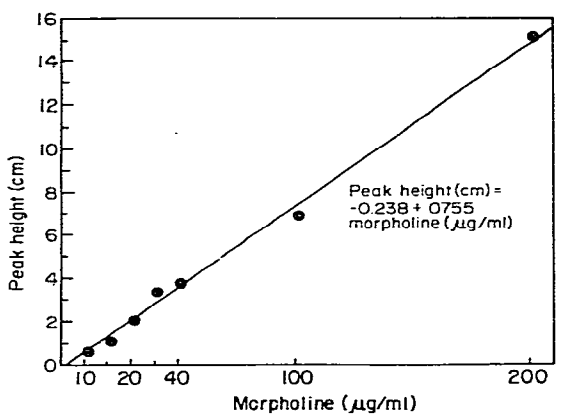

Fig. 2. Standard curve for recovered morpholine.

The recovery of morpholine was: 61% to 70% for plasma, 55% to 58% for urine and 60% to 67.5% for liver tissue.

The precision of the method was checked by using multiple replicates at various concentrations and calculating the standard deviation. The standard deviation ranged from 2 to 8% recoveries.

Results from inhalation experiments using the described method are shown in Table II.

TABLE II

DISTRIBUTION OF INHALED MORPHOLINE IN NZW FEMALE RABBIT TISSUES

Animals were exposed to morpholine vapors (250 ppm for 5 h) by nose exposure. At the end of exposure period the animals were sacrificed and their tissue analyzed for morpholine concentration as described in the text.

Tissue	Morpholine concentration* (μg/g or ml)
Liver	40.6 ± 9.0
Kidney	118.2 ± 6.3
Perirenal fat	10.1 ± 1.8
Lung	40.0 ± 3.9
Spleen	43.9 ± 6.9
Brain	41.9 ± 16.0
Adrenal glands	3.8 ± 0.4
Skeletal muscle	17.8 ± 10
Ovaries	11.2 ± 3.5
Feces	34.7 ± 3.8
Bile	34.0 ± 0.81
Urine	324 ± 86
Blood	20.7 ± 1.30

*Mean ± standard error of the mean (3 experiments).

With this micromethod, plasma, urine, and tissues from animals exposed to morpholine can easily be analyzed with accuracy and precision.

ACKNOWLEDGEMENTS

The author thanks Drs. E.A. Zeller and J.R. Hass for their fruitful suggestions during the development of the method and Ms. J. Schroeder and Mr. W. Gibson for their technical assistance.

REFERENCES

1 M. Greenblatt, S. Mirvish and B.T. So, J. Nat. Cancer Inst., 46 (1971) 1029.
2 J.S. Wishnok and S.R. Tannenbaum, Anal. Chem., 49 (1977) 715A.
3 P. Bannasch and H.A. Mueller, Arzneim.-Forsch., 14 (1964) 805.
4 H. Garcia and W. Lijinsky, Z. Krebsforsch., 77 (1972) 257.
5 P.M. Newberne and R.C. Shank, Food Cosmet. Toxicol., 11 (1973) 819.
6 H. R. Schuller, Amer. J. Path., 89 (1977) 59.