Excretion and Distribution of Morpholine Salts in Rats^{*1}

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Elimination, distribution and metabolism of morpholine salts in rats were investigated by means of chemical analysis and/or radioassay. Gas-liquid chromatography was used for chemical analysis of morpholine in the rat urine and faeces. The analytical results of the excreta accorded with those made by the tracer technique.

When rats were given morpholine-HCl or -palmitate, it was found that about 90% of the dose was excreted in the urine over a period of 3 days and the remaining in the faeces. The faecal excretion of morpholine palmitate appeared to be a little more than that of morpholine-HCl after an oral dose of each labelled compound.

Morpholine was largely excreted unchanged in the urine. The elimination of morpholine from organs, tissues and blood was generally rapid and specific organ-affinity was not observed in other organs except the intestine. The lowest affinity was found for adipose regardless of routes of administration.

Introduction

An emulsion containing morpholine fatty acid salt was used to protect fruit against deterioration. Japanese Standards of Foods Additives still carries this article.

Morpholine may constitute a health hazard as a result of the formation of *N*-nitrosomorpholine in the presence of nitrite in man, since Sander, *et al.*¹⁾ pointed out the formation of carcinogenic nitrosamines in the rat stomach. In fact some authors recently have presented the studies of carcinogenicity of dietary sodium nitrite and morpholine in the rats,^{2,3)} mice⁴⁾ and hamsters.⁵⁾

So far as we know, a little work on the fate of morpholine in mammals has been carried out^{5,6)} and in addition, there is no proper procedure for the quantification of morpholine in

biological samples such as urine, faeces, blood and bile.

In order clearly to know the fate of morpholine, therefore we have developed a sensitive analytical method utilizing gas-liquid chromatography (GLC) that was found to be satisfactory for the determination of free morpholine in the urine and faeces of rats.

This paper reports the elimination of morpholine-HCl and -palmitate in rats using both GLC and radioassay with ¹⁴C-morpholine. The organ-distribution of morpholine-HCl was also studied in the rat with the tracer technique.

Experimental

Chemicals and animals

Morpholine (bp 127~129°C) was used after purification by distillation. The purity was found to be over 99% by GLC. Morpholine-HCl (mp 175~176°C) was recrystallized to give hygroscopic crystals from ethanol. Morpholine-palmitate was prepared from morpholine and palmitic acid in aqueous ethanol. ¹⁴C-Morpholine was synthesized according to the modified method of Maller and Heidelberger⁵⁾ and ex-

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aminated radiochemically. Labelled morpholine was diluted with nonradioactive morpholine to the specific activity of 1.02×10^4 dpm/mg.

Silica gel films (Tokyo Kasei Co.) were used for thin-layer chromatography (TLC) of morpholine and its metabolites. Radioactive compounds were detected by a radioscanner TRM-1B (Aloka). Nonradioactive morpholine was detected as a purple spot on the thin-layer chromatogram by spraying 5% *N*-2,6-trichloro-*p*-benzoquinone imine in dioxane:acetone (4:1).⁷⁾

Radioassay was performed by the same method as previously reported.⁸⁾ Compositions of liquid scintillators were also the same as before.⁸⁾ Following solvents were used for TLC (A) iso-propyl ether-*c*-NH₄OH (12:1), (B) chloroform-*c*-NH₄OH-methanol (18:4:10), (C) chloroform-NH₄OH-acetic acid (143:7:2), (D) ethanol-H₂O-*c*-NH₄OH (100:12:16). *R_f* values of morpholine were 0.64, 0.80, 0.0 and 0.50, respectively, in four solvents described as A, B, C and D.

Male Wistar rats were used in our experiments.

Methods

Extraction and processing of urine and faeces

The collected urine was diluted with 4 volumes of distilled water. The faeces was mixed vigorously with 10 times of distilled water until the homogeneous suspension was obtained. Five ml of each sample was processed as

indicated in Scheme 1.

Analytical method

Morpholine was determined by a GLC (type GC-4APF, Shimadzu) equipped with dual flame ionization detectors on a 2 m × 0.3 cm glass column packed with 30% Apiezon grease M plus 3% potassium hydroxide on 60 to 80 mesh Chromosorb W. The chromatograph was operated at an inlet temperature of 200°C, detector temperature of 230°C, and column temperature of 100°C. The out-flow rate for the carrier gas, nitrogen was 50 ml per minute, and detector gases, air and hydrogen were used with inlet pressure at 0.9 and 0.5 kg/cm², respectively. Two to three μl of sample was injected each time. The retention times of morpholine and cyclohexanone, inner standard, were 4.2 and 8 min, respectively.

When the peak height ratio of morpholine to cyclohexanone was plotted against varying concentrations of morpholine at constant concentration of cyclohexanone, the curve was shown to be linear in the range from 0.1 μg to 1.0 μg. The coefficient of correlation was 0.9996. The calibration curve thus obtained, was used to determine the amount of morpholine.

The sensitivity of detection was calculated on the basis of the minimum detectable peak height for morpholine. The sensitivity limits of this method were determined and found to be 0.1 μg for morpholine, and the precision was about ±5%.

Scheme 1. Preparation of samples for gas chromatographic analysis of morpholine

Animal studies

Elimination study: Male Wistar rats weighing 200 to 350 g were used in the excretion experiment. Rats were fasted overnight only in the case of peroral administration. In the chemical assay of morpholine, dosages of morpholine-HCl were 500 mg/kg for oral administration and 250 mg/kg for intravenous injection. In radioassay, rats were given an oral dose of 200 mg/kg of ^{14}C -morpholine and intravenously at the dosage of about 150 mg/kg, respectively. ^{14}C -Morpholine palmitate was perorally administered to the rat at a dose of 400 mg/kg through a stomach tube. Morpholine-HCl was dosed as a 5% aqueous solution, and palmitate as a dimethyl sulfoxide solution, unless otherwise mentioned.

The animals were held in metabolism cages with free access to water and standard diet (CE-2, Japan CLEA, Tokyo). Urine was collected in the beaker containing 1 ml of $N\text{-HCl}$ and faeces also in the planchet every 24 hours. A water wash of the cages at the end of the experiment was combined with the final day urine.

Distribution study: There were 3 rats weighing 250 to 300 g in each group. This experiment was carried out with ^{14}C -morpholine-HCl

alone. After dosing, the rats were housed in the metabolism cages and allowed free access to food and water. The animals were killed at 2, 6 and 12 hour intervals for the distribution study.

Organs and tissues were removed, dried in air, powdered and weighed. Portions of the samples were oxidized to carbon dioxide for measuring radioactivity in a scintillator. Only the intestine was solubilized in an 0.5 N sodium hydroxide solution under gentle reflux and an aliquot of the solution was mixed with a dioxane scintillator for counting.

The organ-affinity was compared with regard to the level of radioactivity content in terms of RSA (relative specific activity). RSA is defined as the ratio of the activity in the organ per organ weight to the total activity per body weight.

Metabolic studies: The collected urine was extracted with ether or isopropyl ether, and an aliquot of the concentrate was spotted onto thin-layer films, and subsequently developed with several solvents to detect urinary metabolites.

Results

The elimination of morpholine-HCl after its oral and intravenous administration to rats

Table 1. Excretion of Morpholine-HCl in Rats

(hr)	p.o. (%)		i.v. (%)	
	Urine	Faeces	Urine	Faeces
0~24	84.64 $\pm$ 2.64	0.06 $\pm$ 0.02	88.17 $\pm$ 3.02	0.02 $\pm$ 0.01
24~48	4.93 $\pm$ 2.40	0.04 $\pm$ 0.02	1.88 $\pm$ 0.92	0.03 $\pm$ 0.01
48~72	1.22 $\pm$ 0.94	0.04 $\pm$ 0.02	0.49 $\pm$ 0.13	0.03 $\pm$ 0.01
Total	90.80 $\pm$ 0.91	0.14 $\pm$ 0.04	90.55 $\pm$ 2.28	0.08 $\pm$ 0.02

One group consists of 4 to 6 rats. Figures are the mean data $\pm$ S.D.

Table 2. Excretion of ^{14}C -Morpholine-HCl and Palmitate in Rats (p.o.)

(hr)	HCl (%)		Palmitic acid (%)	
	Urine	Faeces	Urine	Faeces
0~24	89.66 $\pm$ 3.56	—	79.40 $\pm$ 1.31	—
24~48	0.89 $\pm$ 0.47	0.61 $\pm$ 0.06	5.73 $\pm$ 2.22	0.84 $\pm$ 0.58
48~72	0.42 $\pm$ 0.12	0.62 $\pm$ 0.12	1.70 $\pm$ 1.00	1.43 $\pm$ 0.24
72~120	—	0.42 $\pm$ 0.09	2.60 $\pm$ 1.55	4.64 $\pm$ 3.08
Total	91.00 $\pm$ 3.85	1.66 $\pm$ 0.13	89.43 $\pm$ 6.08	6.91 $\pm$ 3.90

One group consists of 3 rats. Figures are the mean data $\pm$ S.D.

was very rapid. The results by chemical analysis are shown in Table 1 and agreed well with those of radioassay in Table 2. Over 85% of the dose appeared in the urine during the first 24 hours, while the only slight amount appeared in the faeces. It was found that the urinary excretion after the first 2 days was less than 5% of the dose, and that about 90% of the dose was excreted in the urine over a period of 3 days.

Table 2 shows the comparison of elimination of morpholine-HCl and -palmitate using the tracer technique. Urinary excretion appeared to be much higher in both compounds than excretion in the faeces. Faecal excretion of morpholine palmitate seemed to be a little more than excretion of morpholine-HCl. About 80 to 90% of the dose was excreted in the urine and approximately 3 to 10% in the faeces during 5 days.

Thin-layer chromatography of concentrated urine revealed only the existence of ^{14}C -morpholine which indicated the same R_f values as those of the authentic sample in some solvents. It became clear that morpholine was mainly excreted unchanged in the urine.

The distribution of ^{14}C -activity in the organs at different intervals after dosing is shown in Table 3. The intestine got accumulation of ^{14}C more than any other organs. Significant

quantities of radioactivity were retained in the intestine for the duration of the 12 hours period regardless of administration routes. Small amounts were found in the spleen, heart and stomach at 12 hours after intravenous administration. The blood level of morpholine at 2 hours was reduced to about half the level after 6 hours. The elimination of radioactivity from organs, except the intestine, was very rapid in both cases of intravenous and oral administration.

The pattern of elimination was similar in most organs. The mean value of RSA is expressed in Table 4. The radioactivity of the kidney decreased rapidly from 2 to 6 hours even by different routes. The lowest RSA was observed in the adipose as shown in Table 4. The RSA of the spleen was a little higher in the case of intravenous dosing than in that of peroral administration. Some variation of RSA values was seen among organs, except for the intestine, at 6 hours after dosing, but little at 12 hours.

Discussion

Morpholine, one of secondary amines, is a critical chemical in that this has a possibility to form *N*-nitrosomorpholine, a carcinogen in the presence of nitrite *in vivo*. However, there is no good analytical method applicable to the quantification of morpholine in biological

Table 3. Distribution (%) of ^{14}C -Morpholine in Rats

Organ	2 hr		6 hr		12 hr
	i.v.	p.o.	i.v.	p.o.	i.v. ^{a)}
Brain	0.50±0.10	0.42±0.11	0.09±0.02	0.14±0.03	0.04
Heart	0.11±0.02	0.21±0.09	0.02±0.01	0.04±0.01	0.01
Liver	2.67±0.28	2.51±0.55	0.49±0.16	0.92±0.43	0.19
Lung	0.52±0.21	0.50±0.30	0.05±0.01	0.22±0.02	0.03
Spleen	0.26±0.06	0.29±0.02	0.23±0.23	0.05±0.01	0.01
Kidney	1.47±0.16	1.08±0.26	0.24±0.06	0.44±0.07	0.03
Intestine	28.94±17.27	18.88±9.71	17.37±19.56	13.27±16.65	4.68
Muscle	25.96±12.60	28.01±5.81	2.94±2.09	13.83±2.67	0.15
Adipose	1.50±0.47	1.21±1.56	0.45±3.01	0.52±0.86	n. d.
Testicle	0.78±0.22	0.56±0.09	0.24±0.08	0.21±0.11	0.04
Stomach	0.60±0.21	6.23±5.63	0.10±0.07	2.40±0.69	0.01
Blood	3.20±1.66	2.51±1.70	1.77±0.39	1.26±0.92	0.09

Muscle, adipose and blood were calculated as 40%, 14% and 8% of body weight, respectively.

n.d.=not detected

^{a)} One sample only

Table 4. RSA of ^{14}C -Morpholine in Rats

Organ	2 hr		6 hr		12 hr
	i.v.	p.o.	i.v.	p.o.	i.v.
Brain	0.87	0.69	0.14	0.16	0.06
Heart	0.36	0.54	0.09	0.15	0.02
Liver	0.75	0.60	0.13	0.29	0.07
Lung	1.15	0.74	0.09	0.43	0.03
Spleen	1.52	0.42	0.61	0.32	0.06
Kidney	2.09	1.26	0.33	0.57	0.04
Intestine	4.73	3.45	3.07	3.19	0.87
Muscle	0.63	0.62	0.07	0.33	0.14
Adipose	0.07	0.06	0.05	0.07	n. d.
Testicle	0.85	0.57	0.23	0.25	0.04
Stomach	1.25	9.39	0.17	4.47	0.02
Blood	0.34	0.22	0.27	0.23	0.09

$$\text{RSA} = \frac{(\text{activity in the organ (dpm)}) / (\text{organ weight (g)})}{(\text{total dose (dpm)}) / (\text{body weight (g)})}$$

specimens, although fluorimetric method with dansyl chloride⁹ or 3-carboxy-7-hydroxycoumarin,¹⁰ isotope dilution method,¹¹ and spectrophotometric method with 1,2-naphthoquinone-4-sulphonate¹² have been reported for determination of morpholine in a pure state. Therefore, an attempt has now been made to determine morpholine in biological samples such as urine and faeces. By employing extraction technique, we have successfully applied GLC to the analysis of morpholine. Our developed method possesses advantages over the existing procedures in that color interference is not present and sensitivity is comparable.

Some parameters affecting good recovery and reproducibility were examined in the analytical procedure. Of the solvents tested for the aqueous solution containing morpholine in various concentrations, iso-propyl ether was the most suitable as it has good extraction efficiency and easiness of evaporation before gas-chromatographic analysis. Benzene was also efficient extraction solvent, but was difficult to remove the peak of impurity overlapping that of morpholine on the chromatogram under the running condition.

Extraction of a 0.05% aqueous solution of morpholine with one-half volume of iso-propyl ether gave 95% recovery of morpholine. However, the recovery of morpholine from the urine was 86%. Then, the dilution effect of

the urine with distilled water was investigated but resulted in "without good success." Finally, good recovery was achieved by an increase of alkali concentration. It was noticed in the preliminary tests that the recovery of morpholine varied with the concentration of sodium hydroxide. Therefore, sodium hydroxide solution was added into the urine sample until the highest recovery was obtained.

Eventually, recovery of 98% was found to be able to be obtained by adding 4-fold volume of saturate sodium hydroxide solution to the urine sample (see, Scheme 1). Under such a strongly alkaline condition, morpholine-HCl was converted into free morpholine and it should be quantitatively extracted from the aqueous solution by a single extraction with 0.4 volume of iso-propyl ether. Then the extract was directly subjected to gaschromatography. Recoveries of morpholine present in 1 ml of the blood were studied at two different concentrations of 0.1 and 0.001%. After 5-fold dilution of the blood with distilled water, the determinations of morpholine were undertaken according to the method analogous to the processing for the urine. Average recovery of 3 runs was 89% at 0.1% concentration and 60% at 0.001% concentration.

The bile, on the other hand, gave a low recovery rate of at most 50% at 0.001% concentration. The improvement of low recoveries

was not fully pursued further.

Chemical determination of morpholine in the organs or tissues was much difficult compared with that in the body fluids. Organ-distribution study, therefore, was performed by radio-metric analysis.

From our excretion studies of morpholine salts, it has been shown that the analytical method by GLC is practically sufficient to do the determination of free morpholine in biological fluid. It is clear that morpholine salts are rapidly excreted by rats in both cases of peroral and intravenous administration. The urinary excretion accounts for most of the dose and a small fraction was found in the faeces. The elimination patterns were essentially similar for morpholine-HCl and -palmitate. However, the faecal excretion of the latter seemed to be a little more than that of the former.

Morpholine is excreted almost unchanged in the rat urine and this fact confirms the results on morpholine metabolism by other workers.^{6,7)} Maller and Heidelberger⁹⁾ showed that ¹⁴C-morpholine-HCl on intraperitoneal injection was not metabolized by rats and was eliminated entirely unchanged. Griffiths⁷⁾ also reported the excretion of morpholine after an oral administration of free ³H-morpholine and his experiment resulted in 72 to 83.2% of the ³H appearing in the urine, namely 57.7 to 73.5% on day 1 and only 1.2 to 1.9% in the faeces for 96 hours.

The fact that morpholine is excreted by rats in the unchanged state may be due to its strong basicity (pK_b 9.61) as well known in the literature¹³⁾ which demonstrated no change of highly ionized compounds in the body. Although the presence of the other metabolites can not be ruled out, it would be minor, if any. ¹⁴C-morpholine-HCl on an oral dosing was rapidly absorbed and distributed in many organs. The elimination of radioactivity from the blood and all other organs was very fast, except the intestine. When RSA as an estimate of organ affinity was compared, the extraordinarily high RSA of the intestine was observed at 2, 6 and 12 hours, respectively after injection.

Despite of efforts to clarify the reason of high accumulation in the intestine after mor-

pholine administration, significant amount of morpholine could not be found in the bile. High RSA in the intestine for the oral and intravenous routes is not simply attributable to the bile excretion. Further, the low rate of elimination of morpholine in the faeces seems not to be correlated with its high RSA in the intestine and suggests that morpholine can be reabsorbed before being excreted.

No specific organ-affinity was observed in other organs under experimental conditions. Particularly the lowest affinity was found for adipose in both cases of oral and intravenous administration.

Recently the opening or oxidation of morpholine ring has been reported in metabolic studies of morpholine derivatives.^{14,15)} These findings show that *N*-substituted derivatives of morpholine are metabolized to some extent *in vivo*. However, morpholine itself appears to be an inert substance in the body.

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