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Note

Separation of morpholine and some of its metabolites by high-performance liquid chromatography

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Morpholine is an important industrial chemical with a wide range of applications in the manufacture of pharmaceutical agents, wax emulsifiers, corrosion inhibitors, optical brighteners, rubber chemicals and other products[1]. As a part of our efforts to determine the metabolism and disposition of this compound in rodents we found it necessary to develop efficient methods for the separation of morpholine and some of its metabolites from biological fluids and tissue preparations.

In this communication we report the separation of morpholine, N-hydroxymorpholine, N-methylmorpholine and N-methylmorpholine-N-oxide by two complementary high-performance liquid chromatography (HPLC) systems; one using reversed-phase and the other ion-exchange chromatography. Since both systems can be used at pH values higher than those normally recommended for silica-based HPLC columns, the systems may also have broader application in the separation of various other aliphatic amines without resort to ion-pairing techniques.

EXPERIMENTAL

Morpholine and N-methylmorpholine were obtained from Eastman Kodak (Rochester, NY, U.S.A.), and Aldrich (Milwaukee, WI, U.S.A.), respectively. N-methylmorpholine-N-oxide was provided by Texaco Chemical (Austin, TX, U.S.A.) through the courtesy of N. W. Houghton. N-Hydroxymorpholine was synthesized by the treatment of morpholine with H_2O_2 as described by Henry and Dehn[2]. [U-^{14}C]Morpholine–HCl (specific activity, 20 mCi/mmol, purity > 98%) was obtained from Amersham–Searle Corp. (Arlington Heights, IL, U.S.A.).

Perkin-Elmer Series 3B microprocessor-controlled dual HPLC pumps were used in conjunction with a Rheodyne Model 7125 injector and a Kratos/Schoeffel Model SF 770 variable-wavelength UV monitor. In the first HPLC system, two serially connected 150 × 4.1 mm PRP-1 columns (Hamilton, Reno, NV, U.S.A.) containing 10-μm spherical styrene–divinylbenzene copolymer packing were eluted with a convex gradient (gradient profile "Number 0.3" on the Perkin-Elmer program-

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mer) of 14% to 46% methanol in 0.05 *M* sodium borate, pH 9.2 in 20 min, followed by a 5-min isocratic run at the higher methanol concentration. The flow-rate was 1 ml/min and the UV absorption of the effluent was monitored at 196 nm.

In the second HPLC system, a 300 × 4 mm column packed with Aminex A-5 sulfonic acid type cation-exchange resin in the sodium form, particle size 13 ± 2 μm (Bio-Rad Labs., Richmond, CA, U.S.A.) was eluted with a linear gradient of 0.05 *M* sodium phosphate buffer, pH 6.8, to 0.05 *M* sodium phosphate, pH 10.8, in 20 min at 1 ml/min. The gradient was followed by isocratic elution with the latter buffer for 10 min.

Radioactivity was determined by liquid scintillation counting with quench correction by the external standard-channels ratio method. An Apple II microcomputer was used to convert observed counts per minute to disintegrations per minute.

Animals used in this study were as follows: male Sprague Dawley rats, 250–290 g, male Syrian golden hamsters, 95–135 g and male Strain II guinea pigs, 300–360 g. For the determination of morpholine and its metabolites in urine, each animal was injected intraperitoneally with 125 mg/kg [^{14}C]morpholine (50 μCi) dissolved in saline. After injection, animals were placed in metabolism cages and urines were collected for a period of 24 h in containers thermoelectrically cooled to 0–4°C. To an aliquot of urine, an equal volume of methanol was added, and after clarification of the sample by centrifugation, 100 μl of the supernatant was submitted directly to HPLC. For the analyses of tissue extracts, male Strain II guinea pigs were treated with [^{14}C]morpholine in the same way as above. Four hours after dosing, animals were sacrificed and the livers, the kidneys and the spleens were excised and homogenised in 4 times the volume of 1.15% KCl in 0.01 *M* potassium phosphate buffer, pH 7.5. Aliquots of tissue homogenates were treated in the same manner as the urine samples before submission to HPLC.

RESULTS AND DISCUSSION

Initially morpholine and the three morpholine derivatives dealt with here posed a problem with respect to their detection in the column effluents. Electrochemical detection, as described previously[3], was attempted both in the reductive and oxidative modes but was found to lack sufficient sensitivity. Moreover, no single mode was applicable simultaneously to all four compounds. Although N-hydroxymorpholine can be identified in the column effluent by its characteristic property of reducing $AgNO_3$ to metallic silver[2], this reaction is merely qualitative. As a simple alternative, detection of all four compounds was achieved by monitoring the effluent at 196 nm, which, in general, represents the lower working limit of variable-wavelength UV monitors. Clearly, this method could be applied only to test solutions of standard compounds to establish their elution volumes since the light absorption of the morpholine derivatives, even at this wavelength, is quite low and the presence of many co-eluting UV absorbing compounds in biological samples could interfere with their detection.

As shown in Fig. 1A, the Hamilton PRP-1 columns eluted with a convex gradient of methanol at a constant concentration of borate buffer, pH 9.2, to suppress protonation of the morpholine secondary amine function, achieved a good resolution of all four compounds. Elution was in the order: N-methylmorpholine-N-oxide, N-

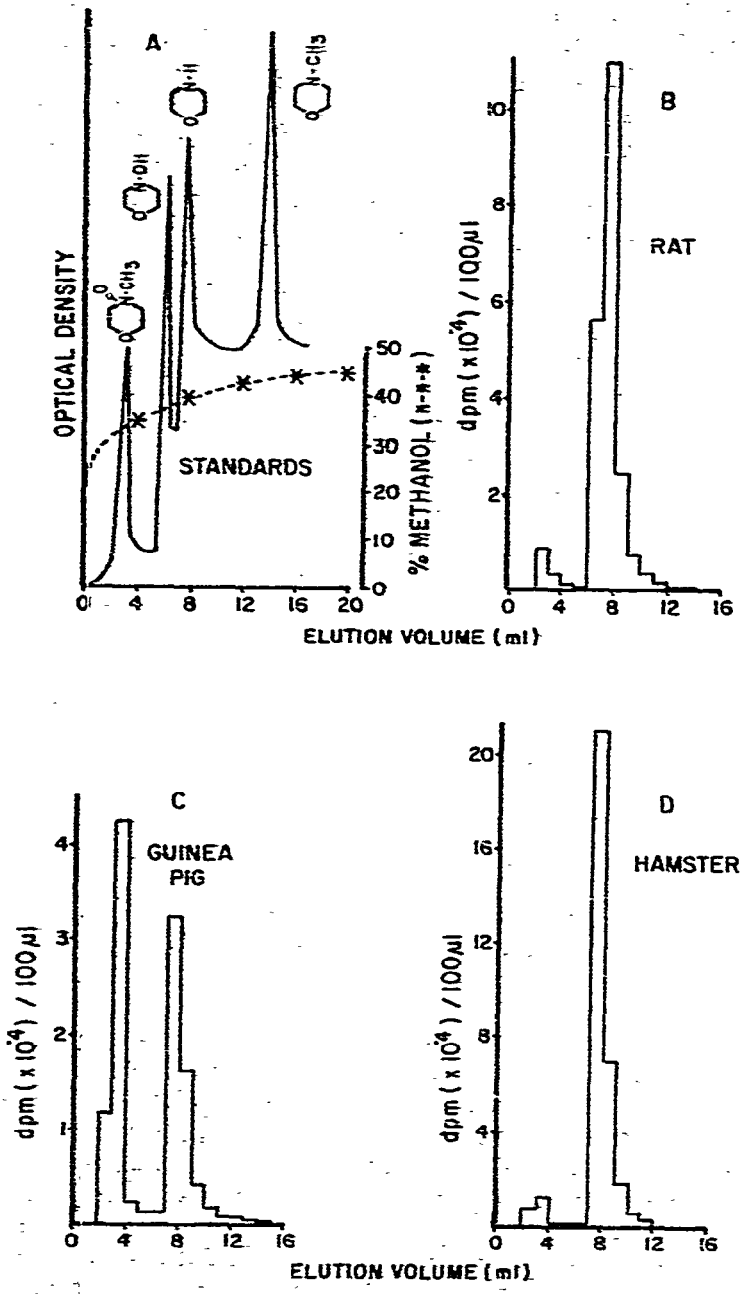

Fig. 1. HPLC profiles of 0–24-h urines from rat (B), guinea pig (C), and hamster (D), treated with [^{14}C]morpholine (125 mg/kg) i.p. Two PRP-1 columns in series were eluted with a convex gradient (dashed lines in A) of methanol in 0.05 *M* sodium borate, pH 9.2. Note upward shift of baseline (A) with increasing methanol concentration.

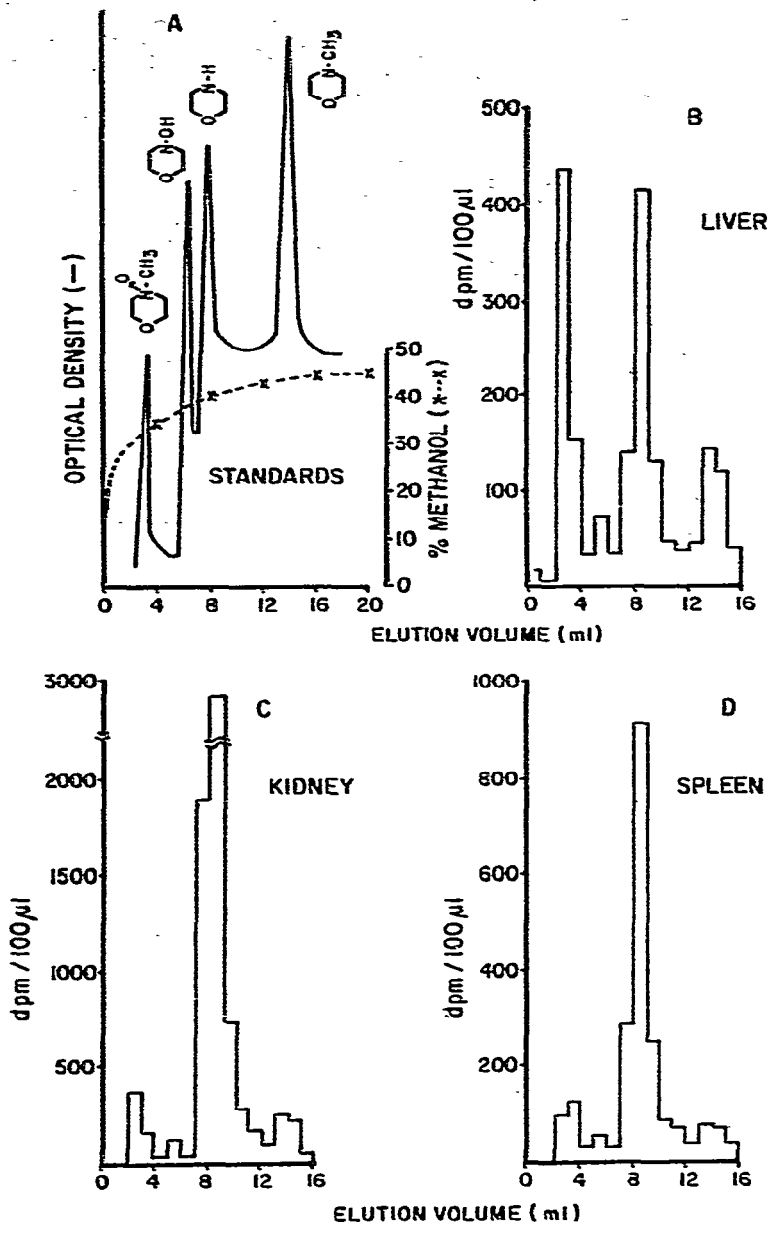

Fig. 2. HPLC profiles of extracts of liver (B), kidney (C), and spleen (D), from a guinea pig treated with [^{14}C]morpholine 4 h prior to sacrifice. HPLC conditions as in Fig. 1.

hydroxymorpholine, morpholine and N-methylmorpholine. Application of this HPLC system to the analyses of urines from rats, guinea pigs and hamsters treated with [^{14}C]morpholine is shown in Figs. 1B, C and D, respectively. In all these cases two peaks of radioactivity were observed: a major peak at 8 ml corresponding to

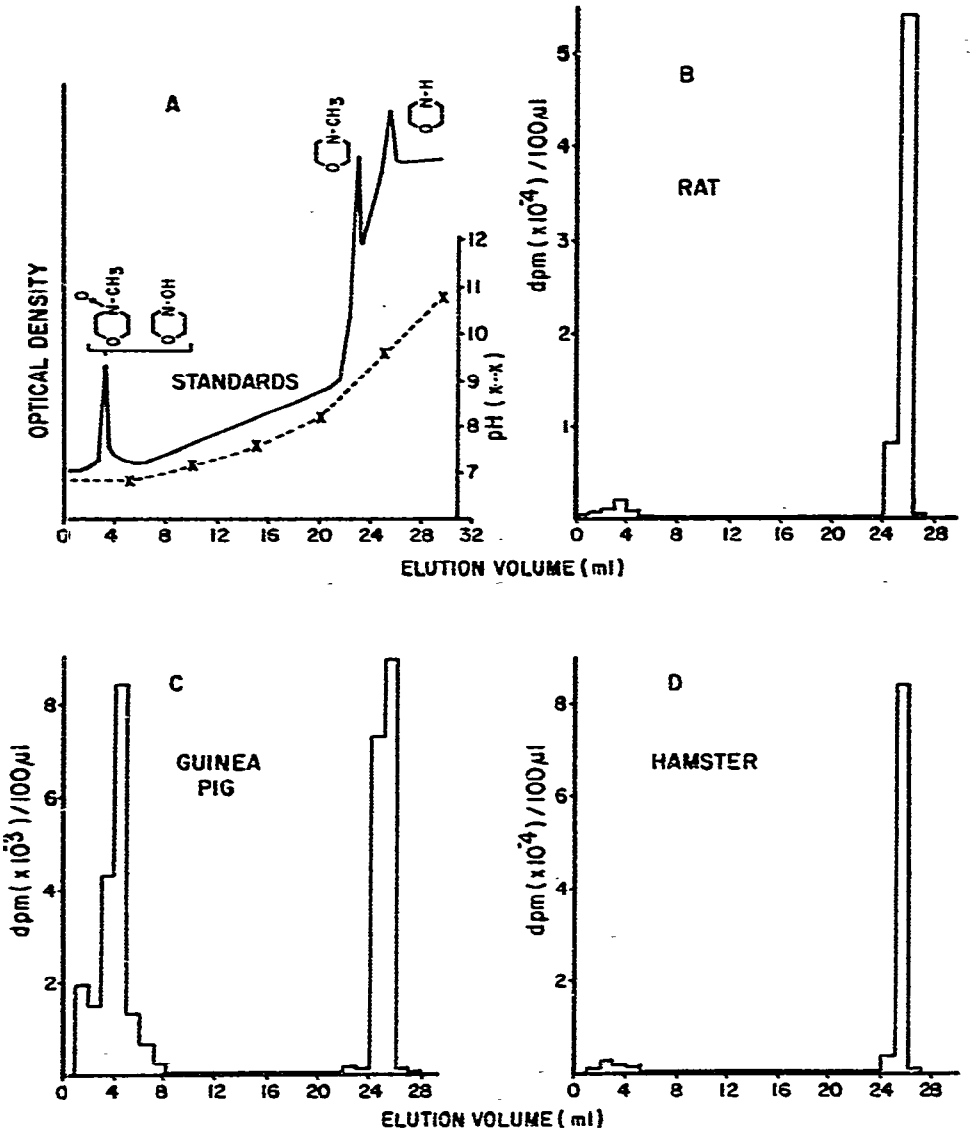

Fig. 3. HPLC profiles of 0–24-h urines from rat (B), guinea pig (C), and hamster (D), treated with [^{14}C]morpholine (125 mg/kg, i.p.). An Aminex A-5 column was eluted with 0.05 *M* sodium phosphate buffer using a pH gradient from 6.8 to 10.8 for 20 min followed by isocratic elution with buffer at pH 10.8. The dashed line shown in A represents the actual pH of the effluent.

unmetabolized morpholine and a second smaller peak at 4 ml corresponding to N-methylmorpholine-N-oxide. The latter formed a considerable portion (20–46%) of the total excreted radioactivity in the case of the guinea pig, but only a small fraction (1–6%) in the case of the rat and hamster.

Hitherto, morpholine has been reported to be resistant to metabolism in experimental animals[4–6]. Thus, the surprisingly large extent of metabolism we ob-

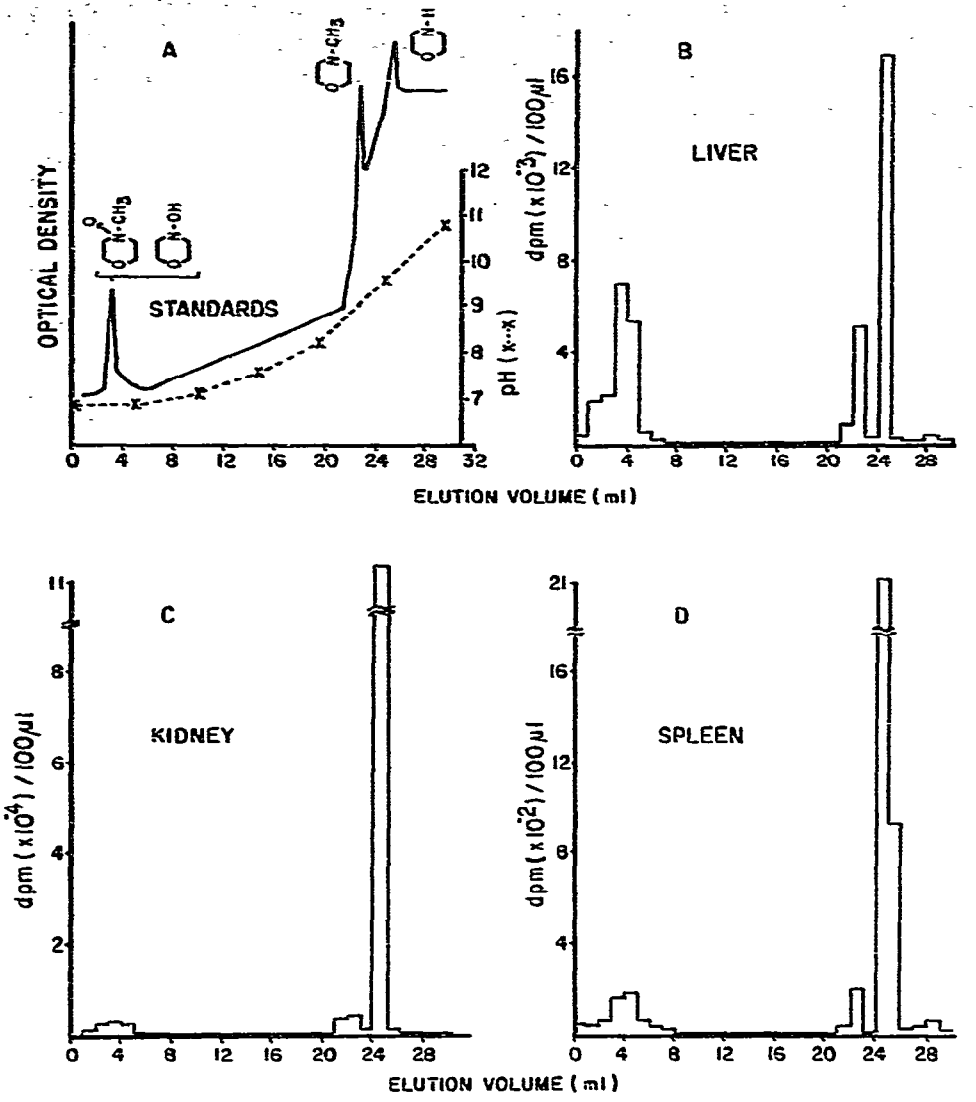

Fig. 4. HPLC profiles of extracts of liver (B), kidney (C), and spleen (D), from a guinea pig treated with [^{14}C]morpholine 4 h prior to sacrifice. HPLC conditions as in Fig. 3.

served in the guinea pig led us to examine several tissues of this species for other possible morpholine metabolites. As shown in Figs. 2B, C and D, extracts of these tissues gave rise in the PRP-1 system to peaks corresponding in elution volumes not only to morpholine but also to N-hydroxymorpholine, N-methylmorpholine-N-oxide and to N-methylmorpholine. The latter is presumably the biological precursor of N-methylmorpholine-N-oxide.

Analogous separations were performed using the Aminex A-5 cation exchange system. While this system failed to resolve N-hydroxymorpholine from N-methylmorpholine-N-oxide (Fig. 3A), morpholine and N-methylmorpholine were well sep-

arated and the order of elution of these compounds was the reverse of that obtained with the PRP-1 system. Again, a substantial amount of N-methylmorpholine-N-oxide was detected in the guinea pig urine (Fig. 3C) in contrast to only small amounts of this metabolite in rat and hamster urines (Figs. 3B and D).

Application of the Aminex A-5 method to extracts of guinea pig liver, kidney and spleen confirmed the presence of N-methylmorpholine in all three tissues (Figs. 4B–D) and gave supporting evidence for the presence of N-methymorpholine-N-oxide. In addition, two minor unidentified peaks at elution volumes of 2 and 29 ml were detected. In a separate work[7] we have described the pharmacokinetics of morpholine in all three rodent species and have confirmed the identity of N-methylmorpholine-N-oxide, isolated from guinea pig urine by Sephadex LH-20 chromatography and thin-layer chromatography, by mass spectrometry.

The two HPLC systems described here are mutually complementary in that they depend on quite different mechanisms (reversed-phase partition and ion exchange) for effecting their separations. Thus, the results so obtained are more reliable and unambiguous than those using only a single mode of HPLC. In addition, the two systems are simpler, less expensive to carry out and much less prone to sample overload than ion-pairing chromatography, the method usually used for the separation of basic aliphatic compounds.

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