# Isolation of Naloxone-3-Ethereal Sulfate from Urine of the Cat[1] (36300)

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Naloxone (Fig. 1) an effective narcotic antagonist in animals (1) and man (2) was recently introduced for the treatment of overdose to heroin and narcotic analgesics. It has also been investigated for managing patients with dependence on heroin (3). Because of these important pharmacologic actions, our laboratory has been involved in isolating major metabolites of naloxone from several species of animals and man (4, 5).

The purpose of the present study is to report on the isolation of a metabolite of naloxone from cat urine, a metabolite not hitherto known to exist in any species. In man (4) and the rabbit (5), naloxone is conjugated in the 3 position with glucuronic acid. In the chicken (5), the metabolite is again a 3-glucuronide but the naloxone portion of the molecule had the 6-keto group reduced to an OH group. Recently Weinstein *et al.* (6) reported that small amounts of this metabolite are also found in man and rabbit. Morphine is conjugated with glucuronic acid in the rabbit (7), dog (8), and man (9, 10) and with sulfuric acid in the chicken (11) and cat (11, 12). Predictions of probable metabolites of naloxone based on extrapolation may be prone to error. For instance, based on our earlier work that the major morphine metabolite in the chicken was the sulfuric ester, one would have expected the analogous ester for naloxone in that species. This expectation would have been wrong on two counts. The major product isolated was the glucuronide and not a sulfate ester. Also, the keto group was reduced. For these various reasons, it was desirable to obtain an unequivocal identification of metabolites in the cat. This paper reports on the isolation of one metabolite in cat urine.

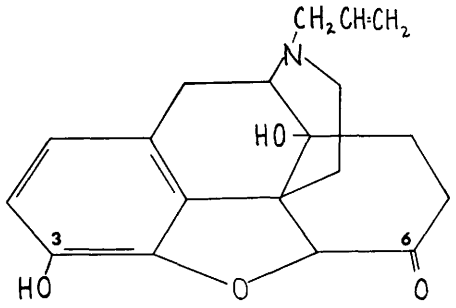


FIG. 1. Formula of naloxone.

*Materials and Methods.* A male cat, weighing 2.2 kg, housed in a metabolic cage, was injected intraperitoneally with 100 mg naloxone HCl at 9 A.M. and 4 P.M. daily until a total dose of 500 mg naloxone HCl was given. Four urine samples of 150, 250, 330, and 430 ml were collected during this 3-day period. The general approach to isolating metabolites was the same as described earlier (4, 5) but essential details are provided below. The urine samples were each processed on an Amberlite XAD-2 resin (Rohm and Haas Company, Philadelphia, PA) column (bed size 1 × 10 in.). The columns were packed with a slurry of resin in water. The urine samples were applied to the columns and each sample was washed through with 10 times its volume of water. The urine and water effluents were discarded. Methanol was then used to elute naloxone and its metabolites. Sixteen 20-ml methanol fractions were obtained from each column. The presence of alkaloid was monitored by thin-layer chromatography. The solvent system was composed of butanol, acetic acid, water (35:3:10, v/v). The absorbant was Gelman Type SG ITLC sheets of silica gel (Fisher Scientific

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Co., Chicago, IL). Alkaloids were demonstrated as purple spots by spraying with iodoplatinate reagent. Organic materials were detected by spraying with sulfuric acid followed by charring on a hot plate. Methanol fractions 2–10 from the four columns were pooled and rotovapored. At this point four iodoplatinate spots were seen on thin-layer chromatograms. The component with the highest mobility on chromatography corresponded to authentic naloxone ($R_F = 0.79$). By far the darkest spot ($R_F = 0.55$) corresponded to the metabolite finally isolated. The two lesser spots had $R_F = 0.37$ and 0.05.

To further reduce the amount of organic contamination, the concentrate in water was applied again to an Amberlite XAD-2 column (dimensions as before) and the column eluted with methanol. Those fractions containing alkaloids were pooled and the solution evaporated under vacuum. Attempts at crystallization by changing to methanol, methanol–acetone mixtures, and acid basic conditions were not successful in producing crystals. A 6-transfer countercurrent distribution was done using 100 ml of each phase of a solvent system consisting of *n*-butanol, water, acetic acid (5:5:1, v/v). The lower aqueous phase was transferred with a syringe equipped with a long 18-gauge needle. The aqueous phases of tubes 4, 5, and 6 were pooled and rotovapored. Again, attempts at crystallization were unsuccessful.

As a further purification, the solution consisting of methanol and lesser amounts of acetone, acetic acid, and water was treated with charcoal ($< 0.5$ g). Sufficient charcoal was added up to a point where obvious decolorization of the solution was obtained. Excess charcoal was avoided in order to prevent large losses of the metabolite. The filtrate was rotovapored to dryness. About 2 ml of hot water was used to dissolve the residue and this solution was left overnight in the cold room. Slightly brownish, needle-like crystals were isolated from the solution by filtration. This 23 mg of crystals was used for spectral and elemental analysis.

*Results and Discussion.* Figure 2 shows the infrared spectrum of the crystallized metabolite along with the spectra of morphine-3-ethereal sulfate and nalorphine-3-ethereal sulfate. Considering the close similarities in the spectra of these three compounds and the broad absorption in the 7.8- to 8.2-$\mu$ range characteristic for ethereal sulfates (11), it was concluded that the metabolite was an ethereal sulfate. Furthermore, the strong absorption at 5.8 $\mu$ for the metabolite (which was absent in the morphine and nalorphine metabolites), indicated that the 6-keto group was intact in the naloxone. That is, this group was not reduced to an OH group (5). To further confirm this last point, a small portion of the metabolite was hydrolyzed in approximately 1 *N* HCl under 15 lb pressure for 1 hr. The hydrolyzate was spotted on a Gelman SG (ITLC) chromatogram sheet, developed in benzene, diethylamine, hexane (25:2:10, v/v) solvent system and sprayed with iodoplatinate. An alkaloid spot with $R_F = 0.83$ was found which was identical to that of authentic naloxone. There was no alkaloid spot corresponding to the reduced naloxone (N-allyl-14-hydroxy-7, 8-dihydronormorphine), $R_F = 0.5$ in this system.

Because our yield of 23 mg of crystalline material was not sufficient to perform carbon and hydrogen analyses, only analyses for nitrogen and sulfur were done. For naloxone ethereal sulfate with 1 molecule of water, $C_{19}H_{21}O_7NS \cdot H_2O$, the calculated percentages were N = 3.3, S = 7.5. The percentage found for the metabolite was N = 3.37 and S = 7.49. The agreement was excellent.

In order to determine whether the sulfuric acid was conjugated to the 3-OH position of naloxone, UV spectra were compared in acid and base; no shift was found. Compounds, such as morphine, which have a free phenolic OH group will show a bathochromic shift in ultraviolet absorption in going from acidic to basic solutions (13). Authentic naloxone showed a shift of the peak from 277 (in HCL) to 290 m$\mu$ (in sodium hydroxide).

These experiments established the structure of one of the metabolites in the urine of the cat. However, the yield of crystalline product was so low that we cannot say whether this is the major metabolite or not.

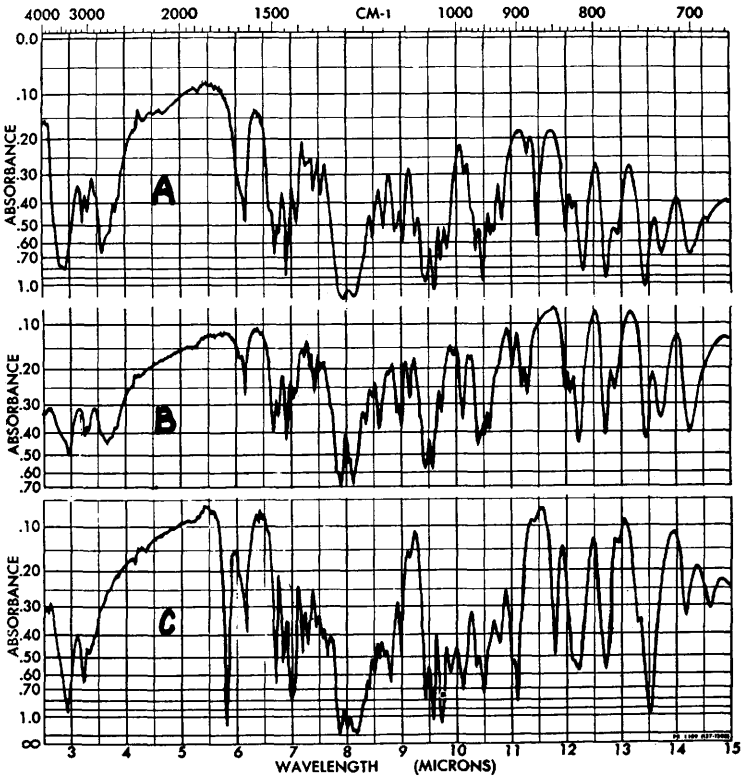


FIG. 2. Infrared spectra (KBr pellet) of (A) morphine-3-ethereal sulfate (B) nalorphine-3-ethereal sulfate (C) metabolite of naloxone isolated from cat urine.

As stated in Methods, the isolated metabolite did correspond to the largest iodoplatinate reactive spot.

*Summary.* A crystalline metabolite of naloxone was isolated from the urine of a cat using column chromatography on Amberlite XAD-2 and countercurrent analysis. Results of infrared and ultraviolet absorption spectra along with elemental analyses established the structure as naloxone-3-ethereal sulfate.

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