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Morphine conjugates in the dog*

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IN VARIOUS animal species, including the human, 3- and 6-glucuronide conjugation,[1–3] 3-ethereal sulfate conjugation,[4, 5] *N*-dealkylation[6–9] and 3-methylation[10] are different metabolic pathways for morphine. Previous studies indicate that the dog, however, metabolizes morphine principally to the 3-glucuronide and presumably a small amount of normorphine (0·2% of the dose), as inferred by the results of collection of pulmonary $^{14}CO_2$.[11] Evidence for some other minor conjugates in the dog has been obtained in the present study.

Morphine-*N*-methyl-^{14}C was injected subcutaneously at a dose of 20 mg/kg to male and female dogs and urine was collected through a catheter. Total drug (free and conjugated) excreted during the first 12 hr was approximately 84·5 per cent of the injected dose. Free drug, estimated by the extraction with ethylene dichloride: *n*-amylalcohol (7:3, v/v) amounted to 13·7 per cent of the injected dose. Urine (240 ml) collected during the 12-hr period was passed through an Amberlite XAD-2 resin† column (2·5 × 25 cm) previously washed with acetone, methanol and water. After adsorption of all radioactivity on the resin, the column was washed with water and eluted with 400 ml methanol until the effluents showed no radioactivity. All methanol eluates were evaporated to dryness under reduced pressure and the residue was triturated repeatedly with small volumes of methanol. After the mixture was centrifuged to separate the insoluble yellowish powder, the methanol supernatant was removed and reduced to a small volume (5–10 ml).

The methanol-insoluble yellowish amorphous powder was dissolved in a small volume of water and passed through a neutral alumina column (1 × 10 cm). The column was washed with water and the resulting light yellow eluate, upon evaporation to dryness under reduced pressure, gave a residue which on recrystallization from aqueous methanol gave colorless crystals of morphine-3-glucuronide dihydrate, whose identity with an authentic sample was confirmed by thin-layer and paper chromato graphy.

The methanol-soluble fraction, on Gelman instant thin-layer chromatography (ITLC) media (silica gel‡) with the solvent system,[5] *n*-butanol–acetic acid–water (35:3:10, v/v), gave six clearly separated radioactive spots of metabolites and morphine with R_f values of: A, 0·0; B, 0·12; C, 0·27; D, 0·46; E, 0·53; and F, 0·83 respectively (Fig. 1). These R_f values varied somewhat on repeated ascending chromatography, but the basic pattern of separation remained the same. Larger quantities of these metabolites were isolated using large (20 × 20 cm) ITLC sheets and eluting the metabolites A, B, C, D and E with hot water. Apparent metabolite F (free morphine) was eluted with hot methanol. Each of the metabolites A, B, C and D gave a positive glucuronide test.[12] The identities of metabolites C and E (Fig. 2) were definitely confirmed as morphine 3-glucuronide and morphine 3-ethereal sulfate, respectively, by descending co-chromatography with authentic samples and by their hydrolysis to morphine on autoclaving in 2·4 N HCl at 15–20 lb pressure for 1 hr.

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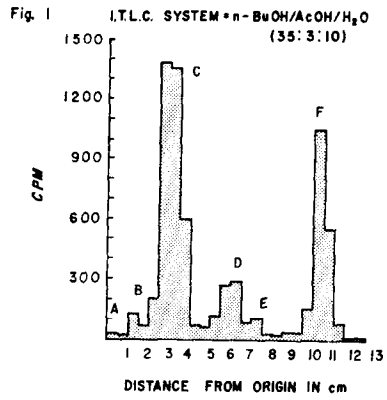
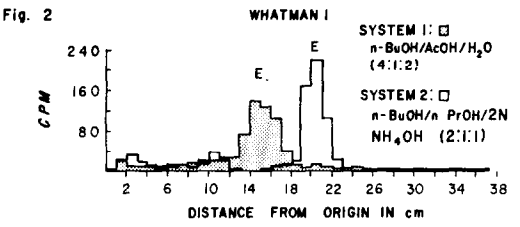
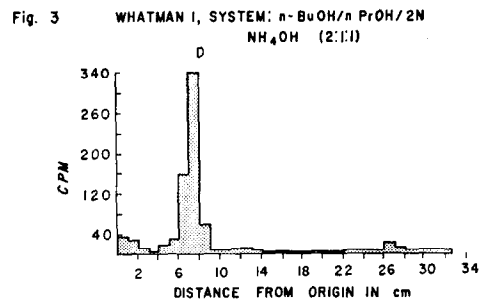
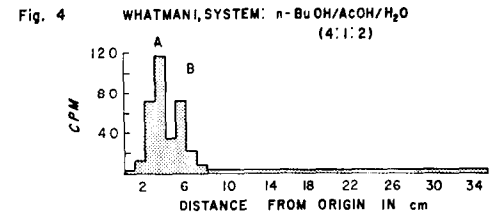
† Amberlite XAD-2 resin, a styrene-divinyl benzene copolymer from Rohm & Haas Co., Philadelphia, Pa.

‡ Uniform glass microfibre sheets impregnated with silica gel obtained from Gelman Instrument Company, Ann Arbor, Mich.









FIGS. 1–4. Radiochromatograms of morphine conjugates in the dog, thin-layer chromatography (Fig. 1) developed by ascending and paper chromatograms (Figs. 2–4) by descending technique.

Metabolite D, rechromatographed on Whatman No. 1 paper with the solvent system, *n*-butanol–acetic acid–water (4:1:2), gave two iodoplatinate-positive spots having R_f values of 0·28 and 0·37, and with the solvent system, *n*-butanol–*n*-propanol–2 N NH_4OH (2:1:1), R_f values of 0·24 and 0·35 respectively (Fig. 3). The ratio of concentration of the first metabolite to the second was approximately 4:1, and only the metabolite with lower R_f values in each of two solvent systems was radioactive. The latter metabolite gave a positive test for free phenolic group (potassium ferricyanide–ferric chloride test). Further, D could be hydrolyzed with β-glucuronidase at pH 7·0 for 24 hr to yield 59·6 per cent of radioactivity as morphine. These results and the chromatographic behavior of this radioactive metabolite indicated that this metabolite was morphine 6-glucuronide, which has been reported to be a minor metabolite of morphine in the rabbit.[3] Due to the nonavailability of authentic material, this point could not be positively settled. Suggestive evidence that the nonradioactive spot was normorphine glucuronide was obtained by hydrolysis and thin-layer chromatography in solvent systems: ethyl acetate–methanol–ammonia (17:2:1) (R_f of authentic morphine and normorphine, 0·90 and 0·67; R_f of experimental morphine and unknown, 0·90 and 0·67) and *n*-butanol–*n*-butyl ether–ammonia (25:70:2) (R_f of authentic morphine and normorphine, 0·73 and 0·35; R_f of experimental morphine and unknown, 0·76 and 0·41) using 0·3% ninhydrin in methanol as spray reagent

Both metabolites A and B gave negative phenolic group tests and, when rechromatographed with the solvent system *n*-butanol–acetic acid–water (4:1:2), gave single radioactive peaks with R_f values of 0·12 and 0·17 respectively (Fig. 4). Hydrolysis of metabolites A and B in 2·4 N hydrochloric acid at 15–20 lb of pressure for 1 hr gave morphine detected by thin-layer and paper chromatography in different systems. Enzymic hydrolysis of metabolite B with β-glucuronidase and glusulase (mixture of β-glucuronidase and sulfatase), chromatography of incubates on Amberlite XAD-2 resin column (1 × 15 cm), elution with methanol, thin-layer chromatography of concentrated eluate and radioscanning showed this metabolite to be practically unchanged. This observation and formation by metabolites A and B of a purple color with ninhydrin may imply conjugation of morphine with some ninhydrin-positive material. Paucity of metabolites A and B precluded further work. Approximate percentages of these metabolites were A, B, 0·5–1·0 per cent; D, 2–4 per cent; E, 1–1·5 per cent. Conjugate C was the major morphine 3-glucuronide, part of which was solubilized in methanol during methanol trituration of the residue from Amberlite XAD-2 column.

Free drug fraction. Apparent metabolite F, eluted from large ITLC sheets with hot methanol or by paper chromatography on Whatman 3 MM sheets with solvent system *n*-butanol–acetic acid–water (4:1:2), was purified by passing the methanolic eluates through a neutral alumina column (1 × 15 cm) and eluting with methanol, colored impurities being held on the column. Concentration of the eluates gave colorless crystals of morphine, the identity of which was confirmed by melting point, thin-layer and paper chromatography in different systems. The methanolic mother liquor did not show the presence of any other metabolite.

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