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RESEARCH ARTICLES

Isolation and Identification of Morphine N-Oxide α- and β-Dihydromorphines, β- or γ-Isomorphine, and Hydroxylated Morphine as Morphine Metabolites in Several Mammalian Species

S. Y. YEH ˣ, H. A. KREBS, and C. W. GORODETZKY

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Abstract □ New morphine metabolites in the urine of guinea pigs, rats, rabbits, cats, monkeys, and humans were isolated with column chromatography, solvent extraction, and TLC and identified with TLC, GLC, and GLC–mass spectrometry. In addition to the known morphine metabolites, morphine N-oxide was isolated from the urine of guinea pigs, and α- and β-dihydromorphines were isolated or detected in the urine of guinea pigs, rats, and rabbits. Monohydroxymorphine was identified tentatively in the urine of guinea pigs, rats, rabbits, and cats. Dihydroxymorphine was identified tentatively in the urine of guinea pigs, rats, and, possibly, rabbits. Finally, β- or γ-isomorphine was identified tentatively in the urine of guinea pigs. The newly described morphine metabolites may be involved in some long lasting pharmacological effects of morphine.

Keyphrases □ Morphine—metabolites isolated and identified in various mammalian species □ Metabolites—of morphine, isolated and identified in various mammalian species □ Narcotic analgesics—morphine, metabolites isolated and identified in various mammalian species

Studies in various laboratory animals and in humans have shown that morphine is metabolized by the following pathways: (*a*) conjugation to give morphine 3-glucuronide, morphine 6-glucuronide, morphine 3,6-diglucuronide, and morphine 3-ethereal sulfate (1–9); (*b*) N-demethylation to yield normorphine, which is conjugated to yield normorphine 3-glucuronide and normorphine 6-glucuronide (6, 10–18); and (*c*) oxidation to form dihydromorphinone (6, 19).

Recently, a minor metabolite, tentatively identified as morphine-2,3-quinone, was reported in the urine of rats administered morphine and after incubation of morphine with rat brain homogenates (20). Also, 2-methoxymorphine was observed in rabbit liver homogenate incubated with morphine and [^{14}C-*methyl*]-S-adenosylmethionine (21). The formation of 2-methoxymorphine may have occurred through hydroxylation of morphine to form a catechol-like compound, which was further methylated. Although O-methylation of morphine to codeine was reported (22, 23), this metabolic pathway was not confirmed (24, 25).

The present paper reports the isolation and identification of free and conjugated morphine N-oxide, free and conjugated α- and β-dihydromorphines[1], and, tentatively, β/γ-isomorphine[1], monohydroxymorphine, and dihydroxymorphine.

EXPERIMENTAL

Drugs—Morphine sulfate, dihydromorphinone hydrochloride, and normorphine hydrochloride were obtained commercially. α-Isomorphine, α-dihydromorphine, and β-dihydromorphine were synthesized as described previously (26). Morphine N-oxide was synthesized according to the procedure of Freund and Speyer (27). [^{14}C-*N-Methyl*]morphine, synthesized according to the procedure of Anderson and Woods (28), had a specific activity of 4 μCi/mg. The purity of the labeled drug was determined with instant TLC impregnated with silica gel[2].

Morphine 3-glucuronide, morphine 6-glucuronide, morphine 3-ethereal sulfate, and normorphine 6-glucuronide were isolated from the urine of humans, dogs, and cats administered morphine (3, 6, 9). Normorphine 3-glucuronide was isolated from the urine of dogs administered normorphine (18). Dihydromorphinone 3-glucuronide and dihydromorphine 3-glucuronide were isolated from the urine of animals administered dihydromorphinone and dihydromorphine, respectively[3]. The characteristics of dihydromorphinone 3-glucuronide and dihydromorphine 3-glucuronide were identical with those reported previously (29).

Animal Experiments—*Short-Term Morphine Administration*—

[1] The alcoholic hydroxyl groups of α- and β-dihydromorphines are at the C-6 position but with different configurations. The hydroxyl groups of morphine and α-isomorphine are at the C-6 position with different configurations, and the double bond is at the C-7–8 position; the hydroxyl groups of β- and γ-isomorphines are at the C-8 position with different configurations, and the double bond is at the C-6–7 position.

[2] Gelman Instrument Co., Ann Arbor, Mich.

[3] Unpublished data.

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Morphine sulfate solution was administered as follows: (*a*) 20 mg/kg sc to two male and two female dogs (8–15 kg), (*b*) 10 mg/kg ip to two male and two female cats (2–4 kg) daily for 3 days, (*c*) 25 mg/kg ip to two New Zealand female and two male rabbits (1.2–3.6 kg) daily for 3 days, (*d*) doses increasing from 12.5 to 50 mg/kg ip to four albino Wistar male rats (300–400 g) twice a day for 4 days, and (*e*) 25 mg/kg ip to five male guinea pigs (0.7–1.0 kg) daily for 28 days. On Days 0, 7, 14, 21, and 28, the same dose of [^{14}C-*N-methyl*]morphine (1 μCi/kg) was substituted for the regular dose of morphine and administered to the guinea pigs by the same route.

After drug administration, animals were individually housed in stainless steel metabolic cages. Urine was collected daily during the entire drug administration period and for 24 hr following the last dose and frozen until drug analysis. Food and water were given *ad libitum*.

Long-Term Morphine Administration—Urine samples were collected from animals and humans chronically administered morphine as follows: (*a*) three male and three female rhesus monkeys[4] (*Macaca mulata*) administered morphine sulfate, 4 mg/kg sc three times a day for at least 1 month; (*b*) two male and two female beagle dogs chronically infused with morphine sulfate for several months in doses up to 500–1000 mg iv/day; and (*c*) four healthy, male, former narcotic addict volunteers stabilized on morphine sulfate, 60 mg sc four times a day for 4 weeks (16).

After drug administration, animals were individually housed in stainless steel metabolic cages. Urine was collected for 24-hr periods and frozen until drug analysis. Food and water were given *ad libitum*. Urine from the human subjects was collected for 24-hr periods by the volunteers and refrigerated until analysis.

Extraction of Morphine and Its Metabolites—Samples with and without hydrolysis were adjusted to pH ~10, buffered with 2 ml of 40% K_2HPO_4, salted, and extracted with 15 ml of 1,2-dichloroethane (glass distilled) containing 30% 2-propanol according to the procedure described by Yeh (16). For determination of morphine *N*-oxide, samples were extracted twice with 10 ml of 1,2-dichloroethane containing 50% 2-propanol (30). After evaporation of the extracts to dryness, the residues were used for TLC, GLC, and GLC–mass spectral identification. The extracts were derivatized with *N*-trimethylsilylimidazole in pyridine[5] in a sealed tube at 90° for 1 hr (16) for GLC and GLC–mass spectral identification.

Hydrolysis of Conjugated Metabolites—Acid hydrolysis and enzymatic hydrolysis of the urine and the isolated metabolites were performed as described previously (16).

Glucuronide and Free Phenol Tests—The glucuronide test was performed according to the procedure of Dische (31). Free phenol was determined by a modified procedure of Volterra (32) with the reagents of Folin and Ciocalteu. (One-tenth of the stated volume of samples and reagents was used.)

TLC—TLC fiber sheets impregnated with silica gel or glass plates precoated with silica gel and an absorbent area[6] were used. The chromatograms were developed either with 1-butanol–acetic acid–water (35:3:10) (Solvent A) or ethyl acetate–methanol–ammonium hydroxide (17:2:1) (Solvent B) and visualized after spraying with iodoplatinate.

In experiments with radioisotopes, TLC sheets were scanned for radioactive spots by cutting the chromatogram into 1-cm strips. These strips were then placed in counting vials, moistened with 0.5 ml of methanol–water (1:1), and evaluated in a liquid scintillation spectrometer[7] after addition of 10 ml of Bray's solution[8].

GLC—A gas chromatograph[9] was equipped with a 0.9-m × 2-mm glass column packed with 3% OV-17 coated on 60–80-mesh Gas Chrom Q. The temperatures of the injector, column, and detector were 255, 220, and 295°, respectively; gas flow rates were 30 ml/min for nitrogen and hydrogen and 300–400 ml/min for dried compressed air.

GLC–Mass Spectrometry—Chemical-ionization mass spectral data were obtained on a gas chromatograph–mass spectrometer[10] equipped with an interactive data system[11] and a 0.9-m × 2-mm glass column packed with 3% OV-17 coated on 60–80-mesh Gas Chrom Q. The temperatures of the injector, column, and ion source were 230, 210, and 100°, respectively. The gas chromatograph was coupled to the mass spectrometer by a 0.31-cm glass-lined stainless steel tube and venting valve.

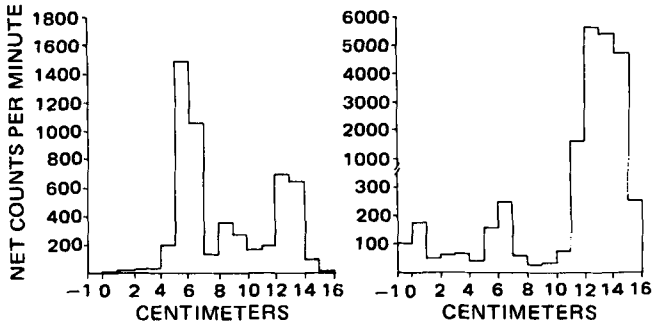


Figure 1—*Radiochromatograms of the organic phase (right panel) showing three spots with* R_f *0.1, 0.4 (as Fraction A), and 0.85 and of the aqueous phase (left panel) showing three spots with* R_f *0.35 (as Fraction M), 0.6 (as Fraction N), and 0.85 (as Fraction O).*

Methane, with a flow rate giving an ion chamber pressure of about 1000 μm, was used as the carrier gas as well as the chemical-ionization reagent gas in the ion source. The electron energy was 80 ev, and the ion repeller voltage was 3 v. Aliquots of 3–5 μl of the silylated sample were injected. After injection, the venting valve was opened for the first 30 sec, allowing excess derivatizing reagent and solvent to escape without entering the ionization chamber.

The obvious GLC peaks were analyzed by examining the mass fragment pattern. In addition, the whole chromatogram was scanned for predicted possible morphine metabolite ions (as silyl derivatives) such as normorphine (*m/e* 416), dihydromorphine (*m/e* 432), monohydroxymorphine (*m/e* 518), dihydroxymorphine (*m/e* 606 and 534) (if one of the four hydroxyl groups was not silanized), codeine (*m/e* 372), norcodeine (*m/e* 358), morphine-2,3-quinone (*m/e* 372), 2-methoxymorphine, and hydroxycodeine (*m/e* 460).

Isolation of Morphine Metabolites in Urine—The 24-hr urine was pooled for animals of the same species receiving the same drug dose. Each 100 ml of the pooled urine was centrifuged to remove the insoluble substances and passed with a flow rate of 2–4 ml/min through a resin[12] column (2.1 × 40 cm) previously washed with methanol and water. The column was washed with 150 ml of distilled water and eluted with 300 ml of methanol containing 2.5% NH_4OH. The effluent was collected in 20-ml tubes.

In experiments with labeled morphine, radioactivity in the urine and the effluent was monitored by counting a 0.5-ml aliquot of each tube with 10 ml of scintillation solution in a liquid scintillation spectrometer. In experiments with nonlabeled drug, morphine and its metabolites in the effluent were monitored with TLC. The methanol eluate, which contained radioactivity or iodoplatinate positive spots, was recombined and evaporated to a syrupy residue with a rotary evaporator under reduced pressure in a water bath at ~50°. (All evaporations were done in this way.) The residue was suspended in 5 ml of 5% NH_4OH solution and extracted three times with 1,2-dichloroethane containing 30% 2-propanol to yield organic and aqueous phases. Several batches of urine were thus processed, and the organic and aqueous phases were pooled for subsequent purification.

The organic phase was evaporated to dryness, and the residue was dissolved in methanol. An aliquot of the methanol solution was chromatographed on instant TLC sheets impregnated with silica gel. The chromatogram was developed with Solvent B and showed three iodoplatinate positive and radioactive spots, R_f 0.0, 0.4, and 0.9 (Fig. 1). A large volume of the methanol solution was chromatographed on large instant TLC sheets impregnated with silica gel (20 × 20 cm). The whole chromatogram was horizontally cut into three sections according to the zones corresponding to R_f 0.0–0.3, 0.3–0.6, and 0.6–1.0.

The substances located in the zone corresponding to 0.0–0.3 were eluted with water; in the zones of 0.3–0.6 and 0.6–1.0, the substances were eluted with methanol. The eluate obtained from the R_f 0.3–0.6 region was marked as Fraction A. The eluate obtained from the R_f 0.0–0.3 region was rechromatographed on instant TLC sheets impregnated with silica gel and developed with Solvent A. The chromatogram showed two spots, R_f 0.35 and 0.85, which were eluted with aqueous methanol and methanol,

[4] Courtesy of Dr. L. S. Harris, Department of Pharmacology, Medical College of Virginia, Richmond, Va.

[5] Tri-Sil-Z, Pierce Chemical Co., Rockford, Ill.

[6] Quantum Industries, Fairfield, N.J.

[7] Mark III, model 6880, Searle Analytic Inc., Des Plaines, Ill.

[8] Composed of naphthalene, 60 g; 2,5-diphenyloxazole, 4 g; 1,4-bis[2-(5-phenyloxazolyl)]benzene, 200 mg; methanol, 100 ml; ethylene glycol, 20 ml; and *p*-dioxane, *q.s.* to 1 liter.

[9] Model 2700, Varian Aerograph, Walnut Creek, Calif.

[10] Model 3300, Finnigan, Sunnyvale, Calif.

[11] Model 6000, Finnigan, Sunnyvale, Calif.

[12] Amberlite, XAD-2 resin, a styrene–divinylbenzene copolymer, Rohm & Haas Chemical Co., Philadelphia, Pa.

Table I—TLC, GLC, and Chemical-Ionization GLC–Mass Spectral Characteristics of Authentic Standards of Potential Morphine Metabolites

Authentic Standard	Instant TLC R_f, Solvent A	Instant TLC R_f, Solvent B	TLC R_f, Solvent B	Phenol Test	Glucuronide Test	As Trimethylsilyl Derivative: GLC R_T, min	GLC–Mass Spectrometry, m/e: $(M-15)^+$	$(M+1)^+$	$(M+29)^+$
Morphine	0.85	0.9	0.4	+	−	5.6	414	430	458
α-Isomorphine	0.85	0.9	0.4	+	−	4.1	414	430	458
Morphine *N*-oxide	0.8	0.4	0	+	−	5.6	414	430	458
Normorphine	0.7	0.8	0.2	+	−	6.8	400	416	444
Dihydromorphinone	0.9	0.9	0.4	+	−	5.6	414	430	458
α-Dihydromorphine	0.8	0.9	0.3	+	−	3.4	416	432	460
β-Dihydromorphine	0.8	0.9	0.3	+	−	3.9	416	432	460
Morphine 3-glucuronide	0.35	0	0	−	+				
Morphine 6-glucuronide	0.5	0	0	+	+				
Morphine 3,6-diglucuronide	0	0	0	−	+				
Morphine 3-ethereal sulfate	0.6	0.4	0	−	−	5.6	414	430	458
Dihydromorphinone 3-glucuronide	0.4	0	0	−	+				
Dihydromorphine 3-glucuronide	0.3	0	0	−	+				
Normorphine 3-glucuronide	0.3	0	0	−	+				
Normorphine 6-glucuronide	0.6	0	0	+	+				
β- and/or γ-Isomorphine[a]				+	−		414	430	458
Monohydroxymorphine[a]				+	−		502	518	546
Dihydroxymorphine[a]							590	606	634

[a] The chemistry and mass spectral data for these compounds are proposed in the absence of authentic standards.

respectively, to yield Fractions B and C. The eluate obtained from the R_f 0.6–1.0 region was rechromatographed on TLC plates, and the chromatogram was developed with Solvent B. The regions corresponding to R_f 0.1–0.3, 0.3–0.5, and 0.5–1.0 were eluted separately with methanol to yield Fractions D, E, and F, respectively.

An aliquot of the aqueous phase was chromatographed on an instant TLC plate impregnated with silica gel. The chromatogram was developed with Solvent A and showed three spots, R_f 0.35, 0.6, and 0.85, corresponding to morphine 3-glucuronide, morphine 6-glucuronide, and free base drugs (morphine, dihydromorphine, and β/γ-isomorphine), respectively (Fig. 1). The aqueous phase, after solvent extraction, was evaporated to a syrupy residue. The residue was triturated with methanol to yield methanol-soluble and methanol-insoluble fractions. The methanol-insoluble fraction was marked as Fraction G. This process was repeated several times to obtain the maximum yield of methanol-insoluble metabolites.

The methanol-soluble fraction was purified by adsorption on 10 g of neutral alumina and added onto a 50-g neutral alumina column (2.5 × 15 cm). The column was eluted with 100 ml of methanol and then with 100 ml each of methanol–water in ratios of 75:25, 50:50, and 25:75. Finally, it was eluted with 500 ml of water, and the effluent was collected in 20-ml tubes. Each tube was monitored for radioactivity in radioisotope experiments (by liquid scintillation spectrometry) or by iodoplatinate positive spots in nonradioactive experiments (shown by TLC). Iodoplatinate positive substances or radioactivity appeared in tubes 8–11 and 24–44. The eluates in these tubes were pooled separately and concentrated. The eluates from tubes 8–11 were purified further on TLC plates developed with Solvent B, as already described, to yield Fractions H–K.

The eluates from tubes 24–44 were evaporated to dryness, and the residue was triturated with methanol to yield methanol-soluble and methanol-insoluble metabolites. The methanol-insoluble metabolites were combined with Fraction G for identification. The methanol-soluble fraction was streaked on large instant TLC sheets, impregnated with silica gel, and developed with Solvent A. The chromatogram showed four spots. The regions corresponded to R_f 0.0–0.1, 0.2–0.5, 0.5–0.7, and 0.7–1.0 and were eluted with water–methanol to yield Fractions L, M, N, and O, respectively.

RESULTS

Identification of Morphine Metabolites in Urine of Guinea Pigs—Approximately 60–70% of the radioactivity present in the urine was recovered in the methanol eluate; 6–10% was recovered in the effluent, including the water washing. The results of glucuronide and free phenol tests and TLC, GLC, and GLC–mass spectral characteristics of authentic standards and of each fraction are presented in Tables I and II.

Identification of Morphine N-*Oxide*—The metabolite isolated in Fraction A was positive in the free phenol test and negative in the glucuronide test and showed the same TLC (Fig. 1), GLC, and GLC–mass spectral characteristics as authentic morphine *N*-oxide. The UV spectrum of this metabolite in alkaline solution showed a bathochromic shift. Furthermore, the metabolite was reduced with sodium bisulfite or titanous chloride to yield morphine (identified by TLC). Based on this evidence, the metabolite was identified as morphine *N*-oxide.

For quantitative estimation of morphine *N*-oxide, the chromatogram was cut into 1-cm strips, and the radioactivity of each strip was measured with liquid scintillation spectrometry after addition of 10 ml of scintillation solution. The amount of morphine *N*-oxide was 2% of the amount of free morphine.

Identification of Free α- and β-Dihydromorphines and β- or γ-Isomorphine—The metabolite isolated in Fraction D was positive in the phenol test. The gas chromatogram showed four peaks (Fig. 2), three of which (peaks 2–4) corresponded in retention times to α- and β-dihydromorphines and morphine, respectively. The mass spectra of peaks 2 (spectrum No. 58) and 3 (No. 65) showed mass ions of 342, 416, 432, and 460, characteristic of dihydromorphine (Fig. 3); peak 4 (No. 96) showed mass ions 340, 414, 430, and 458, characteristic of morphine.

The mass ion of 518, corresponding to the m + 1 ion of monohydroxymorphine, was also seen in the spectrum of peak 4. This compound would be expected to yield a fragment ion of 428 after elimination of one molecule of $(CH_3)_3SiOH$. A small 428 fragment ion was seen in the spectrum of peak 4; however, it could be an isotope mass of the 430 fragment. This peak may be a mixture of morphine and monohydroxymorphine. It is also possible that the morphine may have been a decomposition product of monohydroxymorphine.

The spectrum of peak 1 (No. 48) showed mass ions of 340, 414, 430, and

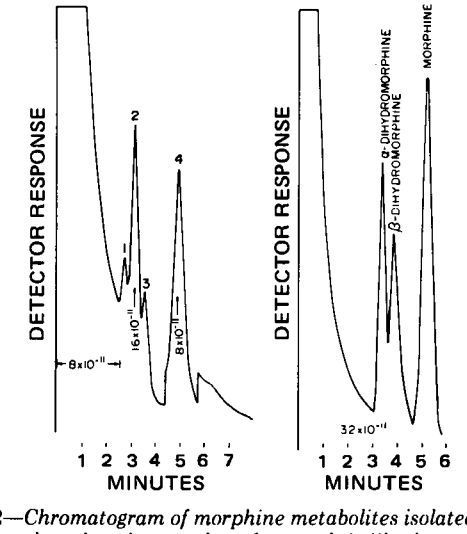


Figure 2—*Chromatogram of morphine metabolites isolated in Fraction D (left) and authentic samples of α- and β-dihydromorphines and morphine (right).*

Table II—TLC, GLC, and Chemical-Ionization GLC–Mass Spectral Characteristics of Morphine Metabolites Isolated from the Urine of Guinea Pig

Isolated Sample	Instant TLC R_f Solvent A	Instant TLC R_f Solvent B	TLC R_f, Solvent B	Phenol Test	Glucuronide Test	As Trimethylsilyl Derivative: GLC R_T, min	GLC–Mass Spectrometry, m/e $(M-15)^+$	$(M+1)^+$	$(M+29)^+$	Identified as
Fraction A	0.85	0.4	0.03	+	−	5.6	414	430	458	Morphine *N*-oxide
Fraction B	0.35	0	0	−	+					Morphine 3-glucuronide
Hydrolyzed B	0.85	0.9	0.4	+	−	5.6	414	430	458	
Fraction C	0.85	0.9	0.4	+	−	5.6	414	430	458	Morphine
Fraction D	0.8	0.8	0.2	+	−	3.0	414	430	458	β-/γ-Isomorphine
						3.4	416	432	460	α-Dihydromorphine
						3.9	416	432	460	β-Dihydromorphine
						5.6	414	430	458	Morphine
								518		Monohydroxymorphine
Fraction E	0.85	0.9	0.4	+	−	5.6	414	432	458	Morphine
Fraction F		0.6–1		+	−	7.1				
Fraction G	0.35	0	0	−	+					3-Glucuronides of:
Hydrolyzed G	0.85	0.9	0.4	+	−	3.0	414	430	458	β-/γ-Isomorphine
						3.4	416	432	460	α-Dihydromorphine
						3.9	416	432	460	β-Dihydromorphine
		0.4				5.6	414	430	458	Morphine, morphine *N*-oxide
		0.7				6.8	400	416	444	Normorphine
Fraction H	0.85	0.4		+	−	4.5				
Fraction I	0.85	0.6–1	0.2	+	−	3.4	416	432		α-Dihydromorphine
						5.6	414	430		Morphine
								518		Monohydroxymorphine
Fraction J	0.85	0.5–1	0.4	+	−	5.6	414	430		Morphine
Fraction K	0.85	0.6–1		+	−	7.1				
Fraction L	0	0	0	−	+					Morphine 3,6-diglucuronide
Hydrolyzed L	0.85	0.9	0.4	+	−	5.6	414	430	458	
Fraction M	0.35	0	0	−	+					3-Glucuronides of:
Hydrolyzed M	0.85	0.9	0.4	+	−	3.0	414	430	458	β-/γ-Isomorphine
		0.7				3.4	416	432	460	α-Dihydromorphine
		0.4				3.9	416	432	460	β-Dihydromorphine
		0.7				5.6	414	430	458	Morphine, morphine *N*-oxide
						6.8	400	416	444	Normorphine
Fraction N	0.6	0	0	+	+					6-Glucuronides of:
Hydrolyzed N	0.85	0.9	0.4	+	−	3.0	414	430		β-/γ-Isomorphine
		0.7				3.4	416	432	460	α-Dihydromorphine
						3.9	416	432		β-Dihydromorphine
						5.6	414	430	458	Morphine
Fraction O	0.85	0.9	0.4	+	−	5.6	414	430	458	Morphine

458, characteristic of morphine. Since the retention time of peak 1 (No. 48) differed from that of morphine and α-isomorphine, it was tentatively identified as β- or γ-isomorphine. Authentic standards of monohydroxymorphine, β-isomorphine, and γ-isomorphine were not available for comparison.

Identification of C-3 Glucuronides of Morphine N-Oxide, Morphine, α- and β-Dihydromorphines, β- or γ-Isomorphine, and Normorphine—The metabolites isolated in Fractions G and M appeared identical and were positive in the glucuronide test and negative in the phenol test. After acid or enzymatic hydrolysis with β-glucuronidase, they were positive in the phenol test. After acid hydrolysis, solvent extraction, and chromatography of Fraction M, the chromatogram on instant TLC sheets, impregnated with silica gel and developed with Solvent B, showed three spots, corresponding to morphine *N*-oxide (0.4); normorphine (0.8); and morphine, β/γ-isomorphine, and dihydromorphine (0.9). The gas chromatogram showed peaks with retention times and mass spectra consistent with α- and β-dihydromorphines, morphine, normorphine, and β- or γ-isomorphine. These results indicate that morphine was biotransformed to morphine *N*-oxide, α- and β-dihydromorphines, normorphine, and, possibly, β- or γ-isomorphine, which were conjugated as glucuronides at the C-3 position.

Identification of C-6 Glucuronides of Morphine and α-Dihydromorphine—The metabolite isolated in Fraction N was positive in the phenol and glucuronide tests, and its R_f value corresponded to morphine 6-glucuronide. After acid hydrolysis or enzymatic hydrolysis with β-glucuronidase, solvent extraction, and chromatography, the chromatogram on instant TLC sheets, impregnated with silica gel and developed with Solvent B, showed one spot, R_f 0.9 (morphine and dihydromorphine). The gas chromatogram showed two peaks with retention times and mass spectra corresponding to α-dihydromorphine and morphine. These results indicate that morphine was biotransformed to α-dihydromorphine 6-glucuronide and morphine 6-glucuronide.

Identification of Morphine 3,6-Diglucuronide—The metabolites isolated in Fraction L were positive in the glucuronide test and negative in the phenol test, and the R_f value was the same as morphine 3,6-diglucuronide previously isolated from human urine (6). The fraction was rechromatographed on instant TLC sheets impregnated with silica gel and developed with Solvent A; the chromatogram showed spots corresponding to morphine 3-glucuronide and morphine. This result may have been due to the instability of morphine 3,6-diglucuronide. A GLC peak was not observed in the fraction prior to hydrolysis. After hydrolysis, a GLC peak with retention time and mass spectra corresponding to morphine was observed.

Tentative Identification of Monohydroxy- and Dihydroxymorphines—A mass ion, *m/e* 518, consistent with the m + 1 ion of monohydroxymorphine was observed in the mass spectrum of the metabolite isolated in Fractions D (Fig. 3) and I (not shown) and the extract of acid-hydrolyzed urine. In addition to this ion, an ion consistent with the mass m + 1 ion of dihydroxymorphine, *m/e* 606, was observed in the extract of acid-hydrolyzed urine (Fig. 4). Masses of 518 and 606 appeared in the spectrum of the same peak. Monohydroxymorphine may be a decomposition product of dihydroxymorphine.

Identification of Morphine Metabolites in Urine of Rabbits—Morphine 3-glucuronide, morphine 6-glucuronide, free and conjugated normorphines, conjugated α- and β-dihydromorphines, dihydromorphinone, and morphine 3,6-diglucuronide were isolated and identified as morphine metabolites in the urine of rabbits administered morphine. Monohydroxymorphine was tentatively identified (Fig. 5).

Identification of Morphine Metabolites in Urine of Rats—Morphine 3-glucuronide, morphine 6-glucuronide, normorphine, dihydromorphinone, and α-dihydromorphine were identified as morphine metabolites in rats (Fig. 6). In addition to the ion of monohydroxymorphine, *m/e* 518, an ion consistent with the m + 1 ion of dihydroxymorphine, *m/e* 606, was observed. A mass of *m/e* 534 also was observed, which could have been due to silanization of only three of the four hydroxyl groups of dihydroxymorphine.

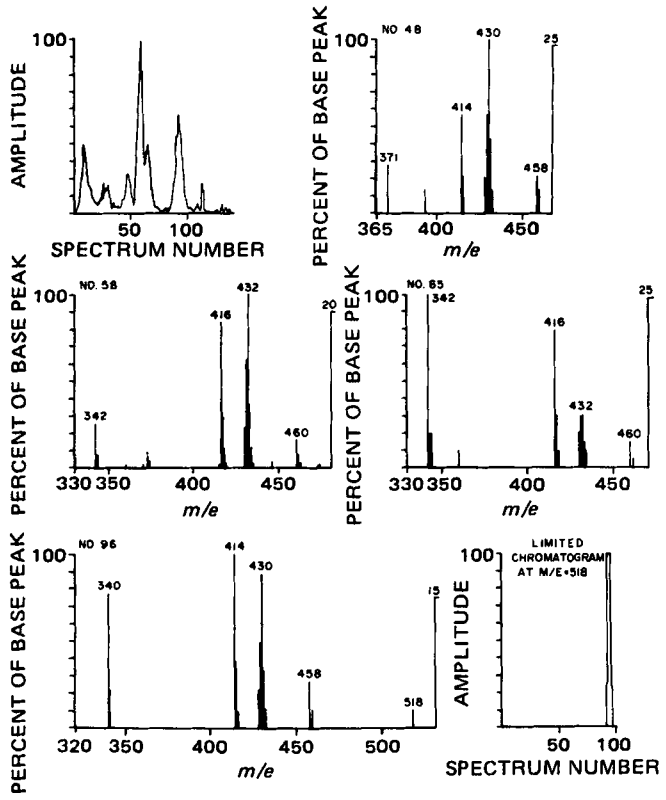


Figure 3—*Integrated total ion current and mass spectra of morphine metabolites isolated in Fraction D. Key: No. 48, β- or γ-isomorphine (tentative); No. 58, α-dihydromorphine; No. 65, β-dihydromorphine; and No. 96, monohydroxymorphine (tentative).*

Identification of Morphine Metabolites in Urine of Monkeys—Morphine 3-glucuronide, morphine 6-glucuronide, normorphine, and dihydromorphinone were identified as morphine metabolites in the urine of morphine-dependent monkeys.

Identification of Morphine Metabolites in Urine of Cats—Morphine 3-ethereal sulfate (a major metabolite), morphine 3-glucuronide, and normorphine were identified as morphine metabolites in the urine of cats (9). Monohydroxymorphine was tentatively identified (Fig. 7).

Identification of Morphine Metabolites in Urine of Dogs—The isolation and identification of morphine metabolites in the urine of dogs

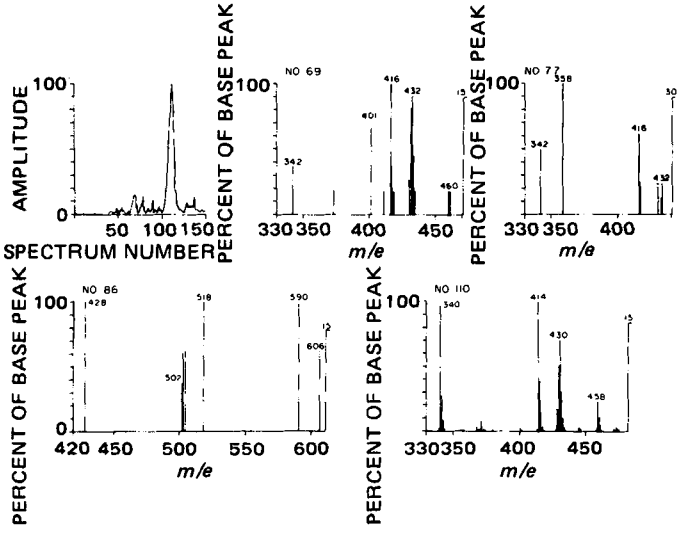


Figure 4—*Integrated total ion current chromatogram and mass spectra of acid-hydrolyzed urine of guinea pigs. Key: No. 69, α-dihydromorphine; No. 77, β-dihydromorphine; No. 86, dihydroxymorphine (tentative); and No. 110, morphine.*

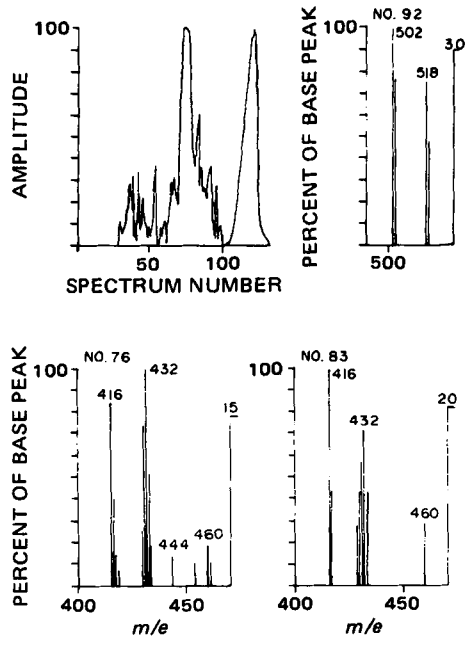


Figure 5—*Integrated total ion current chromatogram and mass spectra of the extract of acid-hydrolyzed urine of rabbits. Key: No. 76, α-dihydromorphine; No. 83, β-dihydromorphine; No. 92, monohydroxymorphine (tentative); and No. 120, morphine (not shown).*

were reported previously (4). In the present study, the extract of acid-hydrolyzed urine was derivatized and screened with GLC and GLC–mass spectrometry for the possible metabolites dihydromorphinone, dihydromorphine, monohydroxymorphine, and dihydroxymorphine. No evidence of these compounds was found.

Identification of Morphine Metabolites in Urine of Morphine-Dependent Humans—The details of a systematic isolation and identification of morphine metabolites in the urine of morphine-dependent subjects were reported previously (6). In the present study, the extract of acid-hydrolyzed urine was derivatized and screened for other possible metabolites. No evidence indicated the presence of dihydromorphine, monohydroxymorphine, dihydroxymorphine, and β- or γ-isomorphine. However, the urine was refrigerated for 3–4 years prior to analysis.

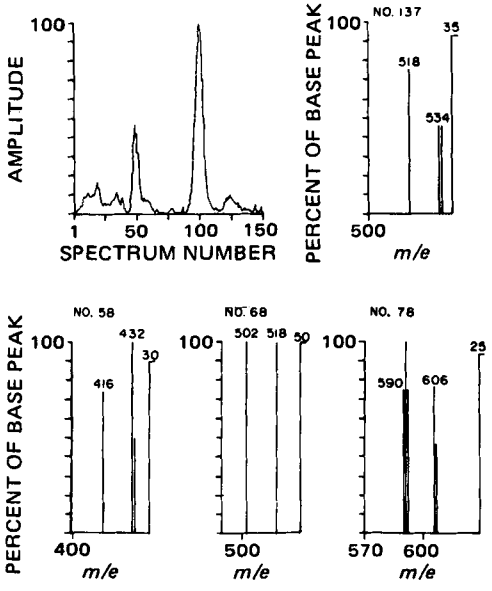
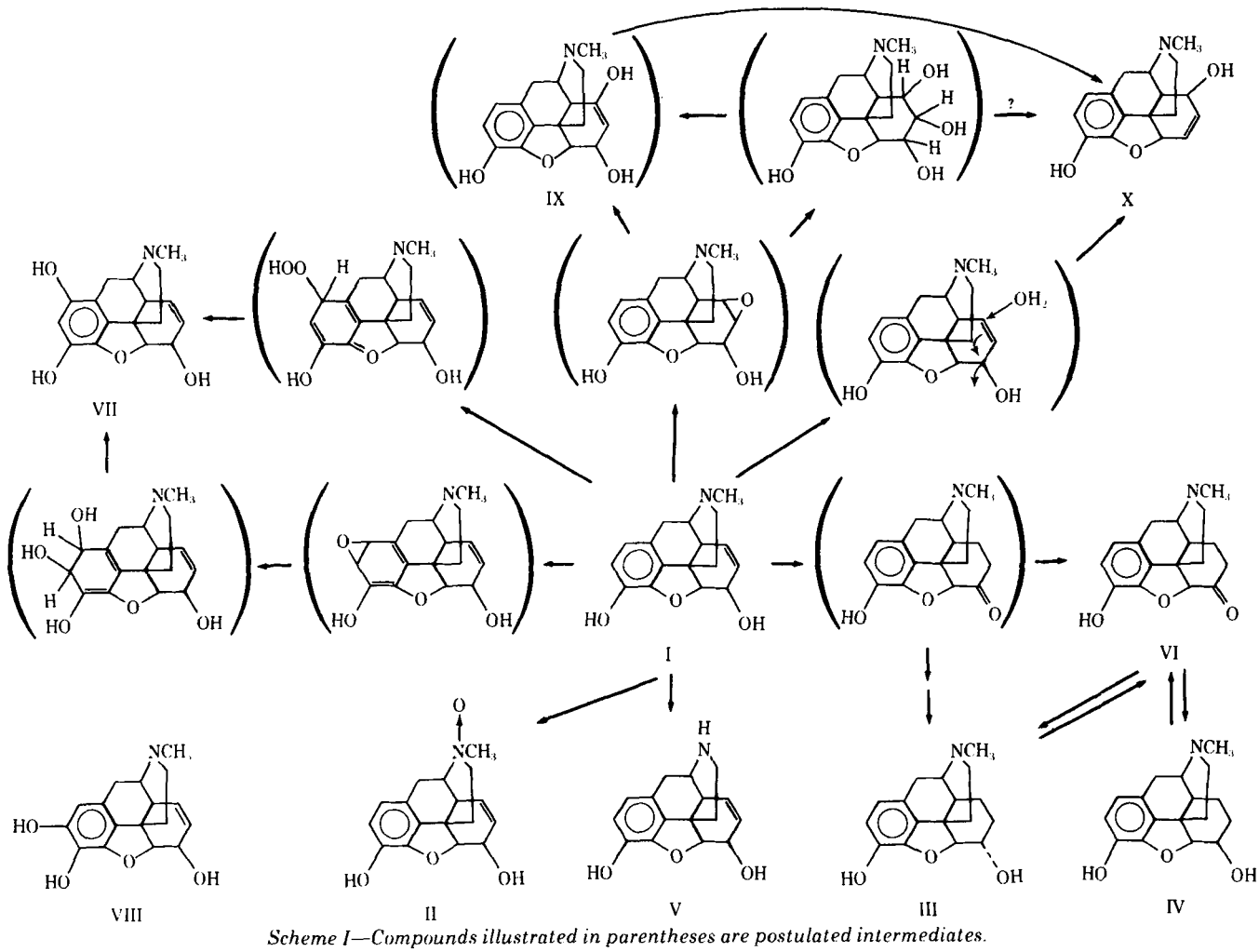


Figure 6—*Integrated total ion current chromatogram and mass spectra of the extract of acid-hydrolyzed urine of rats. Key: No. 58, α-dihydromorphine; No. 68, monohydroxymorphine (tentative); No. 78, dihydroxymorphine (tentative); No. 100, morphine (not shown); No. 125, normorphine (not shown); and No. 137, dihydroxymorphine (assuming only three hydroxyl groups were silanized) (tentative).*



Scheme I—Compounds illustrated in parentheses are postulated intermediates.

DISCUSSION

Although direct evidence is lacking for some steps, it seems reasonable to suggest, based on the metabolism of other compounds, the pathway for the metabolism of morphine (I) shown in Scheme I. Morphine *N*-oxide (II), α-dihydromorphine (III), and β-dihydromorphine (IV), in addition to normorphine (V) and dihydromorphinone (VI), were isolated or detected in the urine. In addition, 1-hydroxymorphine (VII), 2-hydroxymorphine (VIII), 8-hydroxymorphine (IX), dihydroxymorphines, and β- or γ-isomorphine (X) were identified tentatively.

Biotransformation of Morphine to Morphine *N*-Oxide and Normorphine—*N*-Oxide formation, a product of oxidation of a tertiary amine catalyzed by amine oxidase, appears to be a minor metabolic pathway of narcotics. *N*-Oxides of narcotics have been isolated *in vitro* (33–41). The isolation and identification of morphine *N*-oxide in the urine of guinea pigs, therefore, was no surprise. However, it was not isolated and identified in the urine of the other species. Morphine *N*-oxide also might have formed in the body of other species but been reduced in the body or in the urine. Indeed, reduction of morphine *N*-oxide to morphine in rats (30) and of propoxyphene *N*-oxide to propoxyphene in dogs (42) was reported. Furthermore, morphine *N*-oxide may have been present in the urine of the other species at concentrations below the detectable limits of the methods used.

The amine oxidase is present in the liver of pigs, rats, and humans (33–35) and differs from *N*-demethylase. It has a pH optimum at 8.4 and catalyzes oxidation of tertiary amines to their *N*-oxides and secondary amines to hydroxyamines. The *N*-demethylase has a pH optimum at 7.4 and catalyzes oxidation of the *N*-methyl group to form an alcohol and then an aldehyde. The end-product of *N*-demethylation of morphine is normorphine. The factors affecting *N*-demethylase of morphine were discussed previously (16).

Biotransformation of Morphine to Dihydromorphinone and Dihydromorphine—The reduction of the 7,8-double bond of morphine appears similar to reactions occurring in steroid metabolism in the formation of 5α- and 5β-hydroxy metabolites of testosterone, progesterone, and corticoids (43–45). These enzymes do not appear to be substrate specific. Δ′-3-Keto steroids are readily reduced both *in vivo* (43) and *in vitro* (44, 45). Morphine would likely undergo a similar reaction.

Oxidoreductases (dehydrogenases) that reversibly oxidize secondary alcohols to ketones have been evaluated in the study of steroid hormone metabolism. These enzymes can utilize both di- and tripyridine nucleotides as cofactors and do not appear to be substrate specific. Biotransformation of morphine to dihydromorphinone was reported in several species (17, 19). Biotransformation of dihydromorphinone to dihydromorphine was observed in rabbits (29). The reduction of naloxone and naltrexone *in vitro* and *in vivo* to α- and β-naloxols and α- and β-naltrexols, respectively, also was reported (46–53).

Biotransformation of Morphine to Monohydroxy- and Dihydroxymorphines—Ions with a mass *m/e* 518 and 606 equal to the mass of monohydroxymorphine and dihydroxymorphine (as silyl derivatives)

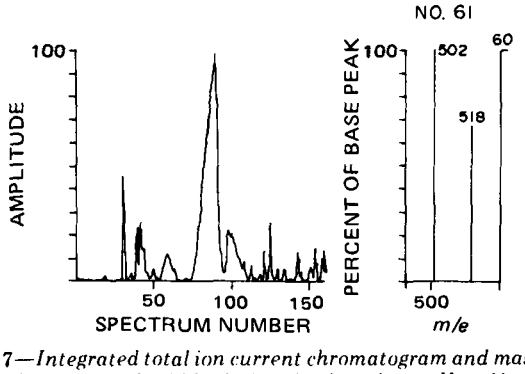


Figure 7—*Integrated total ion current chromatogram and mass spectrum of the extract of acid hydrolyzed urine of cats. Key: No. 61, monohydroxymorphine (tentative); No. 88, morphine (not shown); and No. 100, normorphine (not shown).*

Table III—Morphine Metabolites Identified in the Urine of Several Mammalian Species

Species	Morphine Metabolites[a]											
	XII	V	XIII	XIV	XV	VI[b]	III/IV	X[c]	II	VII/VIII/IX	*m/e* 534[c]	*m/e* 506[c]
Human[d]	+	+	+	+	+	−	−	−	−	−	−	
Guinea pig	+	+	+	−	+	+	+	+	+	+	−	−
Rat	+	+	+	−	±	+	+	−	−	+	+	+
Rabbit	+	+	+	−	±	+	+	−	−	+	+	−
Monkey	+	+	+	−	±	+	−	−	−	−	−	−
Cat	+	+	−	+	−	−	−	−	−	+	−	−
	Minor			Major								
Dog[e]	+	+	+	+		−	−	−	−	−		

[a] XII, morphine 3-glucuronide; V, normorphine; XIII, morphine 6-glucuronide; XIV, morphine 3-ethereal sulfate; XV, morphine 3,6-diglucuronide; VI, dihydromorphinone; III, α-dihydromorphine; IV, β-dihydromorphine; X, β- or γ-isomorphine; II, morphine *N*-oxide; and VII, VIII, and IX, 1-, 2-, and 8-hydroxymorphines, respectively. [b] From Ref. 17. [c] Identifications are tentative. [d] From Ref. 6. [e] From Ref. 4.

were not detected in the hydrolyzed control urine but were seen in the urine extract of some animals administered morphine. It is usual for the chemical-ionization (methane) spectrum of a substance, particularly drugs, to consist only of the m + 1 ion. These compounds were identified tentatively as metabolites of morphine. A paucity of these metabolites and a lack of authentic standards of monohydroxymorphine and dihydroxymorphine for comparison precluded further verification of their identity.

Studies of the metabolism of naloxone and naltrexone, morphine antagonists with structures similar to morphine, in humans and monkeys revealed compounds in the extract of hydrolyzed urine tentatively identified as methoxynaloxone and methoxynaltrexone (as silyl derivatives)[13,14]. In a later study[14], the structures were conclusively shown to be 2-methoxynaloxone and 2-methoxynaltrexone as compared with authentic samples. The formation of 2-methoxy derivatives of morphine, nalorphine, levorphanol, and phenazocine was observed in rabbit liver microsomes (21). The formation rate of the catechol-like compound appears to be correlated with the lipid solubility of the substrate. A similar biotransformation of morphine (*i.e.*, hydroxylation) might have occurred and resulted in the mass spectra detected in the extract of urine of some animals, as already noted.

Hydroxylation of morphine could be taking place in the aromatic as well as in the aliphatic ring. 2-Hydroxy-, 10-hydroxy-, and 14-hydroxymorphines, 14-hydroxydihydromorphine, 14-hydroxycodeine, and 14-hydroxycodeinone have been synthesized (55–59). The hydroxylation of the aromatic ring at position 1 could proceed either by the well-known epoxide pathway or by the hydroperoxide pathway, as in butylated hydroxytoluene metabolism (60). Since morphinedihydrodiol was not detected, the second pathway appears to be more attractive, although the epoxide pathway cannot be ignored. One hydroxyl group of dihydroxymorphine might be in the aromatic ring while the other one is in the aliphatic ring.

The tentatively identified monohydroxy- and dihydroxymorphines might be the end-products of morphine metabolism *via* an epoxide pathway. The epoxide can bind covalently to macromolecules, *e.g.*, receptor sites, protein, or other molecules and might be released slowly from the body. The possible metabolic intermediates or the reaction product between intermediates and a macromolecule or a degradation product of that adduct might be responsible for some long-term effects of morphine. Protracted abstinence observed in humans and animals (61–63), prolonged tolerance (64), and a decrease in growth rate of offspring of morphine-treated rats and mice (65–67) might be due to the intermediate metabolite(s) of morphine bound to the macromolecule.

Biotransformation of Morphine to β- or γ-Isomorphine—Two possible pathways could lead to the conversion of morphine to isomorphine: hydroxide-ion direct attack at the C-8 position or formation of morphine 7,8-epoxide. Attempts to synthesize morphine 7,8-epoxide by modifying the procedure of Craig and Purushothaman (68) and Kishi *et al.* (69) failed. TLC of the reaction product showed two spots. The R_f of the major spot corresponded to that of morphine *N*-oxide. A small peak with mass consistent with monohydroxymorphine, in addition to morphine, was observed in the reaction product. Possibly, the 7,8-epoxide is a transient intermediate and is converted to isomorphine spontaneously. Further *in vitro* studies are needed to verify these suggestions.

Glucuronides of Morphine, Morphine *N*-Oxide, Dihydromorphinone, and Dihydromorphine—Glucuronidation is a universal metabolic pathway for biotransformation of foreign compounds having a phenolic or alcoholic hydroxyl group. Conjugation of morphine, dihydromorphine, normorphine, and dihydromorphinone with glucuronic acid at the 3- and/or 6-positions has been reported. The finding of glucuronides of morphine *N*-oxide and α- and β-dihydromorphines in the present studies was expected. Chromatograms of the methanol effluent or the aqueous phase after solvent extraction of the methanol effluent (obtained from the urine of rats, rabbits, and monkeys) on instant TLC sheets, impregnated with silica gel and developed with Solvent A, showed a spot with R_f 0.0–0.1, corresponding to that of morphine 3,6-diglucuronide. No attempt was made to establish the identity of this fraction.

Ethereal Sulfate Conjugation of Morphine—The major metabolite of morphine in cats was morphine ethereal sulfate; morphine glucuronide was a minor metabolite. There is a deficiency of glucuronyl transferase in the cat, but this enzyme is present in major quantities in the other species.

SUMMARY

Morphine metabolites isolated or detected in the species studied are summarized in Table III. Normorphine, morphine 3-glucuronide, and morphine 6-glucuronide were found in humans, guinea pigs, rats, rabbits, monkeys, dogs, and cats. In cats, morphine 3-glucuronide was a minor metabolite and morphine 6-glucuronide was not observed. Morphine ethereal sulfate was observed in humans and cats. Dihydromorphinone was observed in guinea pigs, rats, rabbits, and monkeys. Both α- and β-dihydromorphines were observed in guinea pigs, rats, and rabbits.

Morphine *N*-oxide and β- or γ-isomorphine were observed in guinea pigs. Hydroxylated morphine was observed in guinea pigs, rats, rabbits, and cats. In these studies, the metabolism of morphine in guinea pigs was investigated more thoroughly than in the other species using radioactively labeled drug. The minor metabolites found in guinea pigs but not in other species might also be present in the urine of other species but not in a sufficient concentration to be detected by the methods used.

Codeine, norcodeine, methoxyhydroxymorphine (2-hydroxycodeine), and morphine-2,3-quinone were not detected.

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