Isolation and Characterization of Urinary Metabolites of Nalorphine in Dogs and Cats

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Abstract □ Nalorphine-3-glucuronide dihydrate and nalorphine-6-glucuronide were isolated as urinary metabolites of nalorphine in dogs, and nalorphine-3-ethereal sulfate and nalorphine-3-glucuronide dihydrate were isolated in cats. These metabolites were characterized by UV and IR spectra, phenolic test, nalorphine and glucuronic acid determinations, elemental analyses, and enzymatic hydrolysis.

Keyphrases □ Nalorphine, urinary metabolites—isolation, characterization, in dogs, cats □ TLC—separation □ Paper chromatography—separation □ UV spectrophotometry—identification □ IR spectrophotometry—identification

Nalorphine is *N*-dealkylated to normorphine *in vitro* by rat liver microsomes (1) and *in vivo* in rats (2) and cats (3), and it is conjugated in rats (4) and dogs (5). Recently, nalorphine-3-glucuronide and nalorphine-3-ethereal sulfate were isolated from the urine of rabbits and cats (6), respectively. The present studies describe the isolation and characterization of these and other nalorphine metabolites from the urine of dogs and cats injected with nalorphine.

METHODS

Urine of female dogs injected with 20 mg./kg. s.c. of nalorphine HCl (total of 2.1 g.) was collected with a catheter up to 24 hr. after the last injection and frozen immediately after each collection. Urine of two male cats injected three times daily, each with 50 mg./kg. s.c. of nalorphine HCl (total of 1.0 g.), was cage-collected up to 48 hr. after the last injection and frozen immediately after each collection.

Isolation and Purification of Nalorphine Metabolites in Dogs—The procedure used for the isolation and purification of nalorphine metabolites was similar to that used for isolation of morphine-3-glucuronide from the urine and bile of rats injected with codeine (7); only the details essential to the method are given here. The urine sample (450 ml.), separated from insoluble substances, was loaded onto an ion-exchange (Amberlite XAD-2) resin[1] column. The column was washed with water (250 ml.) and eluted with methanol (400 ml.). A portion of the methanolic eluate (150–400 ml.) was passed through an ion-exchange (Amberlite IRC-50) resin[1] column, and the column was washed with methanol (50 ml.). Free alkaloids were adsorbed by the resins, whereas conjugated metabolites passed through them.

Isolation of Conjugated Metabolites—The effluent and washing from the Amberlite IRC-50 column were concentrated at 55° under a stream of N_2. White crystals of nalorphine conjugate were separated from the methanolic solution, which was finally evaporated to a viscous residue. The residue was triturated with methanol and recrystallized from an aqueous methanol solution. These crystals and those separated from methanolic effluent during concentration were chromatographed on instant thin-layer chromatography (ITLC)-type silica gel[2] and paper and showed the same R_f values in different solvent systems. Therefore, these crystals were combined, recrystallized from boiling water, washed with methanol, and dried at 100° *in vacuo* for 24 hr. to yield snow-white crystals (1.02 g., 55% of injected nalorphine). The purity of these crystals, called nalorphine Metabolite I from dogs, was established by paper chromatography and ITLC.

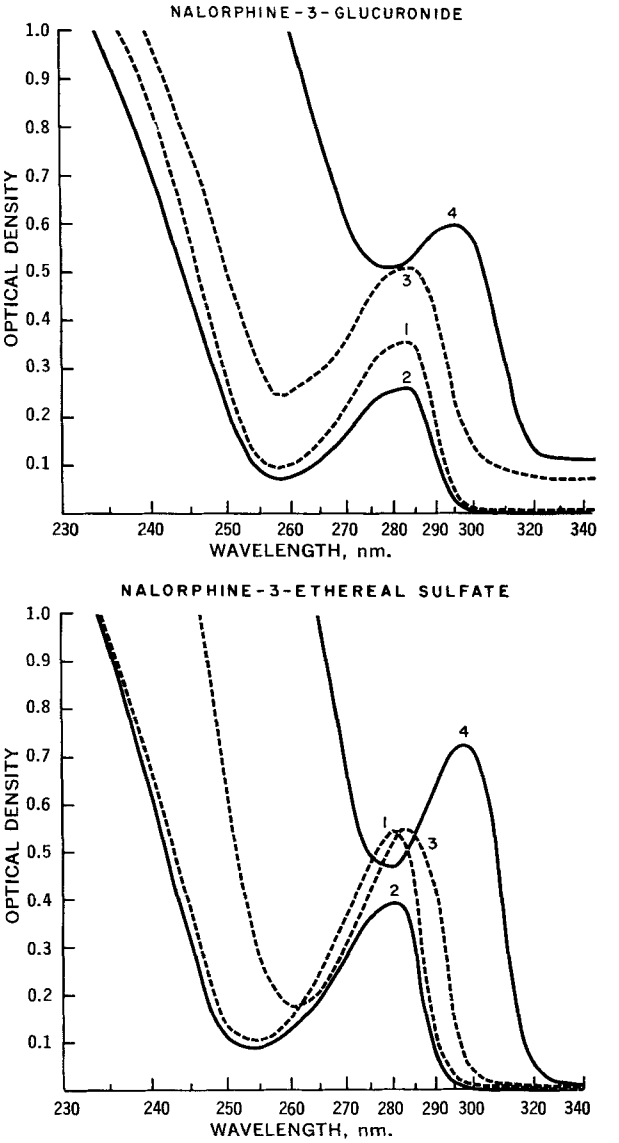

Figure 1—*UV spectra of nalorphine-3-glucuronide and nalorphine-3-ethereal sulfate. Key: Curve 1, 2.5 ml. of nalorphine-3-glucuronide or nalorphine-3-ethereal sulfate in H_2O (1 mg./10 ml.) and 0.5 ml. of 1 N HCl; Curve 2, 2.0 ml. of the metabolite solution and 1.0 ml. of 1 N NaOH solution; Curve 3, 3 ml. of the acid-hydrolyzed metabolite in HCl solution; and Curve 4, the acid-hydrolyzed metabolite in NaOH solution.*

Paper Chromatographic Separation of Conjugated Metabolites—The aqueous methanolic mother liquor remaining after crystallization of Metabolite I was evaporated to dryness. This residue was dissolved in about 10 ml. of water and passed through a neutral aluminum oxide column (1 by 10 cm.). The column was eluted with water (300 ml.), colored impurities being held on the column. Evaporation of the eluate to dryness gave a slight yellowish residue,

[1] Rohm & Haas Co., Philadelphia, Pa.
[2] Gelman Instrument Co., Ann Arbor, Mich.

Table I—R_f Values of Nalorphine Metabolites Isolated from Dog and Cat Urine[a]

Substance	Paper Chromatography[b]		TLC[b]		
	n-BuOH–AcOH–H_2O (4:1:2)	*n*-BuOH–H_2O(9:1)[c]	*n*-BuOH–AcOH–H_2O (35:3:10)	EtOAc–MeOH–NH_4OH-(30%NH_3) (17:2:1)	*n*-Butyl Ether–*n*-BuOH–NH_4OH(30%NH_3) (70:25:2)
Nalorphine-3-glucuronide (Metabolite I)	0.36	—	0.41		
Nalorphine-6-glucuronide (Metabolite II)	0.40	—	0.62		
Nalorphine-3-ethereal sulfate(Metabolite III)	0.50	—	0.69		
Residue of free alkaloid fraction					
a, from the dog urine	0.58; 0.68	0.13; 0.95	0.9	0.95	0.24; 0.81
b, from the cat urine	0.58; 0.68	0.13; 0.95	0.9	0.95	0.24; 0.81
Authentic normorphine	0.58	0.13	0.9	0.83	0.24
Authentic nalorphine	0.68	0.95	0.9	0.98	0.81

[a] Chromatograms were sprayed with potassium iodoplatinate solution or 3% ninhydrin in methanol. [b] *n*-BuOH = *n*-butanol; AcOH = acetic acid; EtOAc = ethyl acetate; MeOH = methanol. [c] Paper buffered with citrate phosphate solution at pH 6.8.

which was dissolved in 1 ml. of water and chromatographed (5 μl./spot) on Whatman 3 MM paper (19 by 22 in.) with *n*-butanol–acetic acid–water (4:1:2 v/v) as the solvent system. The chromatograms showed two clearly separated zones having R_f values: Zone A, 0.36; and Zone B, 0.40. These bands were separated and extracted with 100–200 ml. of boiling water. Each extract was evaporated to dryness, and the residue was recrystallized from an aqueous methanol solution. The substance extracted from Zone A, obtained in crystalline form, was identical with Metabolite I, whereas the substance from Zone B (called Metabolite II) could not be crystallized.

The Amberlite IRC-50 column was eluted with 200 ml. of ammoniacal (30% NH_3)–methanol (1:1), and the eluate was evaporated to dryness. Free nalorphine and normorphine were identified in the residue by ITLC, using *n*-butyl ether, *n*-butanol, and NH_4OH (30% NH_3) as the solvent system, and by paper chromatography on Whatman 3 MM paper buffered with citrate phosphate solution at pH 6.8 (8) and developed with *n*-butanol and water (Table I).

Isolation of Nalorphine Metabolites from Cat Urine—By application of a procedure similar to that used for dog urine, the major nalorphine metabolite (205 mg.) was obtained as colorless crystals (called Metabolite III, 23% of injected nalorphine).

The minor metabolite (called Metabolite IV, 20 mg., 2.3% of injected nalorphine) was isolated by paper chromatography from the methanolic mother liquor as described previously. Metabolite IV was identical with Metabolite I and characterized as nalorphine-3-glucuronide.

Free nalorphine and free normorphine were identified in the free alkaloid fraction as already described.

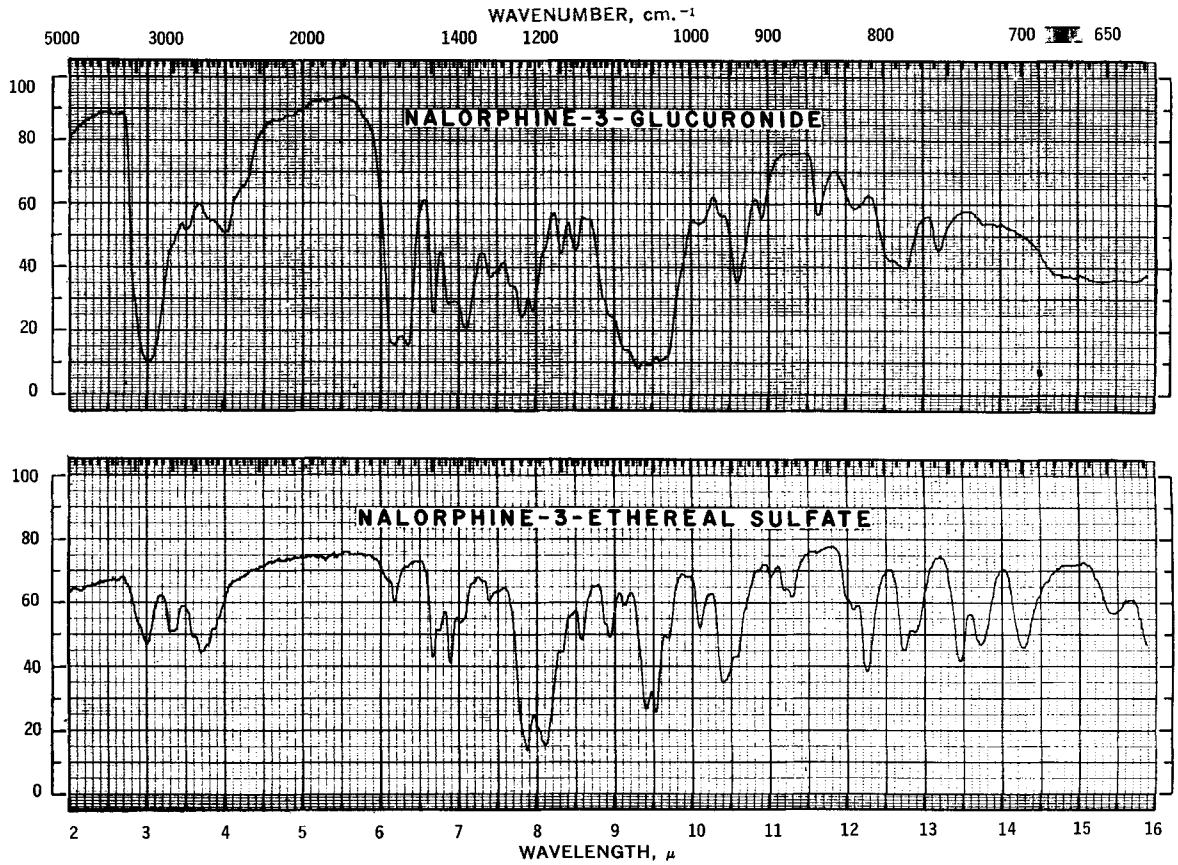

Figure 2—*IR spectra of nalorphine-3-glucuronide and nalorphine-3-ethereal sulfate.*

Table II—Physicochemical Characteristics of Urinary Nalorphine Metabolites

Characteristic	Metabolite I	Metabolite II	Metabolite III
Solubility	Sparingly soluble in H_2O; insoluble in usual organic solvents		Sparingly soluble in H_2O; insoluble in usual organic solvents
Melting point	237–238° dec.		>300°
Phenolic test[a]	Negative	Positive	Negative
UV spectra(Fig. 1):			
In HCl and H_2O, max. nm.	285	285	280
In NaOH, max. nm.	285	295	280
IR spectra (Fig. 2)	3.0 μ (polyhydroxy), 6.1 μ (ionized carboxylic acid)		7.8 and 8.1 μ (ethereal sulfate)
Acid hydrolysis	+[b]	+	+
Enzymatic hydrolysis:			
With β-glucuronidase[c]	+	+	−
With glusulase[d]			+
With phenol sulfatase[c]			+
Elemental analyses[e]	Found (%) Calcd. for $C_{25}H_{29}NO_9 \cdot 2H_2O$	Found Calcd.	Found (%) Calcd. for $C_{19}H_{21}NO_6S$
	C, 57.36 C, 57.35		C, 58.6 C, 58.29
	H, 6.54 H, 6.35		H, 5.13 H, 5.13
	N, 2.57 N, 2.57		N, 3.47 N, 3.58
			S, 8.09 S, 8.20
Content of nalorphine[f]	60.0 59.47	62.0 63.87	78.5 79.4
Glucuronic acid[g]	38.5 37.08	38.5 39.82	

[a] Sequential treatment with alkaline ferricyanide and acid ferric chloride solution. [b] + indicates nalorphine obtained on hydrolysis. [c] Obtained from Sigma Chemical Co., St. Louis, Mo. [d] Obtained from Endo Lab., Garden City, N.Y. [e] Performed by the Department of Chemistry, University of Iowa, and Huffman Lab., Wheatridge, Colo. [f] By methyl orange method (9). [g] By carbazole method (10).

RESULTS AND DISCUSSION

Chromatographic Studies—Chromatographic data on ITLC and paper chromatograms of nalorphine Metabolites I, II, and III are presented in Table I. These R_f values varied somewhat on repeated chromatography, but the basic pattern of separation remained the same.

Characterization of Nalorphine Metabolites—The physicochemical characteristics of the nalorphine metabolites are listed in Table II. Based on these data, nalorphine Metabolite I, isolated from the urine of dogs after subcutaneous injection of nalorphine, was characterized as nalorphine-3-glucuronide dihydrate. Metabolite II was identified as nalorphine-6-glucuronide. Nalorphine Metabolite II on ITLC and paper chromatograms had higher R_f values than nalorphine-3-glucuronide. Previous studies (11, 12) also reported that morphine-6-glucuronide had higher R_f values than morphine-3-glucuronide. It is probable that an unknown nalorphine metabolite observed by Fujimoto *et al.* (6) in the urine of rabbits is nalorphine-6-glucuronide on the basis of similarity of chromatographic behavior observed in the present study.

Nalorphine Metabolite III, identified as nalorphine-3-ethereal sulfate, was reported to be a metabolite of nalorphine in the cat (6). Nalorphine-3-glucuronide, a minor metabolite of nalorphine in the cat, was also observed in the cat urine by Fujimoto *et al.* (6) but was not isolated.

Chromatographic evidence for the presence of a small amount of normorphine (0.1% of injected dose) was obtained in the urine of dogs and cats.

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