Pharmacokinetics of Morphine and Its Surrogates I: Comparisons of Sensitive Assays of Morphine in Biological Fluids and Application to Morphine Pharmacokinetics in the Dog

EDWARD R. GARRETT ˣ **and TÜRKAN GÜRKAN** *

Received December 5, 1977, from *The Beehive, College of Pharmacy, J. Hillis Miller Health Center, University of Florida, Gainesville, FL 32610.* Accepted for publication March 2, 1978. *Present address: Department of Analytical Chemistry, Faculty of Pharmacy, University of Istanbul, Istanbul, Turkey.

Abstract □ A sensitive isotope derivatization assay was developed to quantify morphine in biological fluids in the nanogram per milliliter range. Morphine, derivatized with ^{3}H-dansyl chloride, was separated from the reaction products by TLC. The spots were scraped from the plate, and the eluted radioactivity was determined by liquid scintillation. The standard deviations of this morphine assay were ±18.6 ng/ml in 100 μl of plasma and ±1.86 ng/ml in 1 ml of plasma. The GLC analysis of pentafluoropropionated morphine in the range of 0–5 ng of morphine/ml of plasma had a standard deviation of ±0.46 ng/ml when 1 ml of plasma was taken. Liquid scintillation spectrometric analysis of ^{14}C-morphine had a sensitivity of 1.5 ng/ml of plasma at double the background. There were no significant differences among the liquid scintillation, electron-capture GLC, and radioisotope derivatization methods for morphine obtained from the plasma of a dog given 14.00 mg iv of morphine. Morphine conjugates were assayed as morphine after the acid hydrolysis of plasma and urine preextracted to remove unconjugated morphine, and the equivalence of various methods was demonstrated to monitor plasma and urine pharmacokinetics in a dog.

Keyphrases □ Morphine—radiochemical TLC analysis in biological fluids, compared to GLC and liquid scintillation spectrometric analyses, pharmacokinetics in dog □ Radiochemical TLC—analysis, morphine in biological fluids, compared to GLC and liquid scintillation spectrometric analyses □ Pharmacokinetics—morphine in dog, radiochemical TLC, GLC, and liquid scintillation spectrometric analyses compared □ Narcotic analgesics—morphine, radiochemical TLC analysis in biological fluids, compared to GLC and liquid scintillation spectrometric analyses, pharmacokinetics in dog

The disposition and pharmacokinetics of morphine have been studied in humans and animals (1–12). Assays with sufficient sensitivity to determine morphine at nanogram per milliliter levels in biological fluids used radioactive (13–16), spectrofluorometric (17–21), and GLC (22–29) techniques. Procedures utilizing radiolabeled morphine are highly sensitive but are relatively nonspecific unless accompanied by chromatographic separation. Fluorometric methods have high sensitivity but lack specificity and have high interfering backgrounds. Radioimmunoassay measures picogram amounts of morphine but also assays metabolites and analogs (30–32).

It is necessary to derivatize the polar morphine molecule for reliable GLC assay. Morphine has been converted to its trimethylsilyl and acetyl derivatives and quantified by flame-ionization detection (22–26, 29). Methods utilizing GLC–mass fragmentography with a practical lower limit of detection of 500 pg were reported (33–35). Electron-capture GLC assays of morphine derivatized with trifluoroacetic anhydride (27) and with pentafluoropropionic anhydride (28) were applied to biological samples.

In preparation for detailed studies on the pharmacokinetics of morphine and its surrogates, this paper reports the comparison of the modified electron-capture GLC assay of pentafluoropropionic anhydride derivatized morphine (28), a new radioisotopic derivatization technique using ^{3}H-dansyl chloride, and selected extraction techniques for liquid scintillation assay of *N*-methyl-labeled ^{14}C-morphine in biological fluids. The methods were applied to a pharmacokinetic study of morphine in the dog.

EXPERIMENTAL

Materials and Methods—Spectral grade benzene[1], 1-butanol[1], ethyl acetate[1], toluene[1], chloroform[1], isopropyl alcohol[1], acetone[1], and methanol[1] were used. A toluene-based scintillation cocktail[2] was used in the radioactive assays. Reagents were pentafluoropropionic anhydride[3], silylating fluid[4], ^{3}H-dansyl chloride in benzene solution[5] (8.3 Ci/mmole and 1 mCi/ml), and dansyl chloride[6]. High purity hydrochloric acid[7], sodium hydroxide[7], sodium carbonate[7], and sodium bicarbonate[7] were used to prepare buffer solutions.

N-Methyl-labeled ^{14}C-morphine[5] (60 mCi/mmole) and morphine sulfate crystalline[8] USP were used to prepare the dose (3.85 mCi/mmole) for the pharmacokinetic study. ^{14}C-Urea[9] (9.76 mCi/mmole) was the internal standard for the radioactive assay. Precoated silica gel plates[10] on aluminum supports were used for TLC separations. Nalorphine[8] was the internal standard for GLC assays.

Apparatus—The gas chromatograph[11] was equipped with a ^{63}Ni-electron-capture detector. The GLC separation was carried out with a silylated 3.05-m coiled glass column[12] at 215° with a detector temperature of 275°. The carrier (nitrogen) flow rate was 30 ml/min.

Radioactivity of the samples was determined with a liquid scintillation counter[13].

A chromatograph[14] equipped with a 6000-psi constant-flow pump and a 254-nm detector was used for high-pressure liquid chromatographic (HPLC) separation of morphine. The column[15] was designed for reversed-phase chromatography.

GLC Assay—An aliquot (0.5 ml) of plasma or urine was placed in a 15-ml centrifuge tube containing 1.5 ml of freshly prepared 1 *M* sodium bicarbonate adjusted to pH 8.9 with 1 *M* NaOH. An appropriate amount (2–10 μl) of the internal standard, nalorphine, in 0.1 *M* sulfuric acid was added and was usually between 3 and 350 ng. The amount of nalorphine was chosen to give a peak area ratio of the separated morphine pentafluoropropionate to nalorphine pentafluoropropionate between 0.5 and 2. Prior radioactivity measurements of radiolabeled morphine permitted the best estimates of the appropriate amounts of nalorphine to add.

The mixture was thoroughly mixed with 2 ml of benzene–1-butanol (85:15) on a vortex mixer for 30 sec. The phases were separated by centrifugation[16] for 10 min at 3000 rpm, and the organic phase was transferred with a silylated Pasteur pipet into another 15-ml centrifuge tube. The extraction was repeated with 2 ml of solvent. The aqueous phase was saved for subsequent assay of morphine conjugates.

[1] Burdick and Jackson Laboratories, Muskegon, Mich.
[2] Scinti Verse, Fisher Scientific Co., Fair Lawn, N.J.
[3] Reactivial, Pierce Chemical Co., Rockford, Ill.
[4] Silyl-8, Pierce Chemical Co., Rockford, Ill.
[5] Amersham/Searle Co., Arlington Heights, Ill.
[6] Aldrich Chemical Co., Milwaukee, Wis.
[7] J. T. Baker Chemical Co., Phillipsburg, N.J.
[8] Merck & Co., Rahway, N.J.
[9] Bionuclear Corp., Sun Valley, Calif.
[10] E. Merck Laboratories, Elmsford, N.Y.
[11] Model 3920-B, Perkin-Elmer, Norwalk, Conn.
[12] Supelco Inc., Bellefonte, Pa.
[13] Model LS 330, Beckman Instruments, Fullerton, Calif.
[14] Model ALC 202, Waters Associates, Milford, Mass.
[15] μBondapak C_{18} Porasil, Waters Associates, Milford, Mass.
[16] Model K, International Centrifuge, Needham Heights, Mass.

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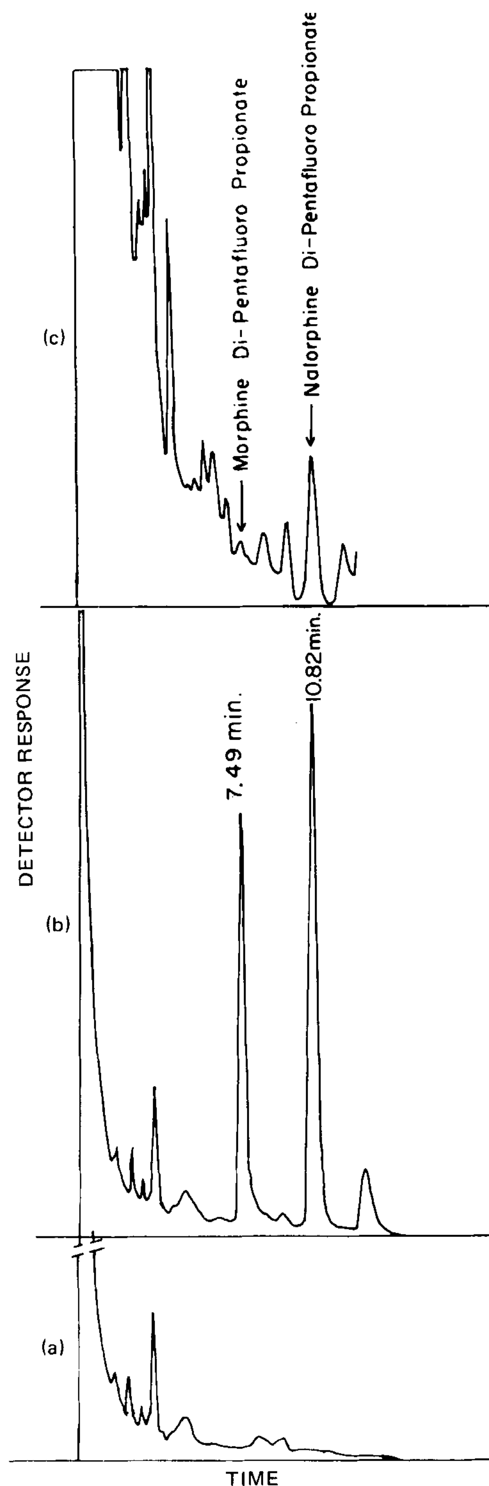

Figure 1—*Electron-capture GLC analysis of pentafluoropropionyl-derivatized morphine and nalorphine from dog plasma. The chromatograms represent the injection of 1 of 200 μl of the final solution from a derivatized extract of 1 ml of blank plasma (0 ng/ml) with an attenuation of 64 (a), the injection of 1 of 500 μl of the final solution of derivatized compound from 0.5 μl of plasma (525 ng/ml) taken 1.36 min after injection of 0.91 mg/kg iv as morphine base but with an attenuation of 128 (b), and the injection of 1 of 50 μl of the final solution of derivatized compound from 1 ml of plasma (1.32 mg/ml) taken 420 min after injection with an attenuation of 64 (c).*

The combined organic phases, containing morphine and internal standard, were back-extracted into 0.5 ml of 1 *M* HCl by mixing on a vortex mixer for 30 sec. The phases were separated by centrifugation for 10 min at 3000 rpm, and the organic phase was aspirated and discarded.

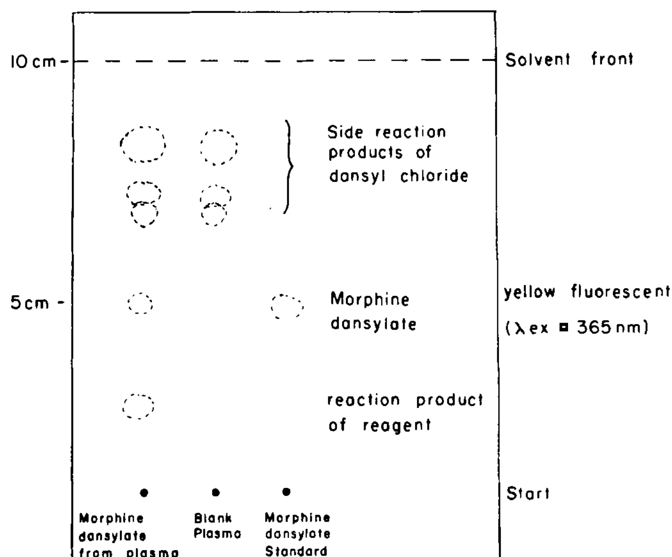

Figure 2—*Thin-layer chromatogram of morphine dansylate prepared from morphine extracted from plasma compared to a derivatized extract of blank plasma and pure morphine dansylate.*

The aqueous phase was washed with 2 ml of fresh organic solvent, and the solvent was aspirated and discarded. The acid phase was neutralized with 0.5 ml of 1 *M* NaOH, the pH was adjusted to 8.9 with the 1 *M* carbonate buffer, and the solution was twice reextracted as already described with 2 ml of benzene–1-butanol.

The organic phases were combined in a vial[3] (5 ml) and evaporated under a nitrogen stream at 55° on a heating block[17]. The thoroughly dried residue was derivatized with 100 μl of pentafluoropropionic anhydride in 50 μl of ethyl acetate for 30 min on a heating block at 55°. Excess reagent was evaporated under a nitrogen stream. The thoroughly dried residue was dissolved in 50 μl of toluene, and 1 μl of this solution was injected into the gas chromatograph. This solvent gave much less of a solvent peak than the previously used ethyl acetate (28).

Quantification was based on the peak area ratio of morphine pentafluoropropionate and nalorphine pentafluoropropionate. The peak areas were measured by an integrator[18]. Typical chromatograms of pentafluoropropionic esters of morphine and nalorphine extracted from plasma are given in Fig. 1. Linear calibration curves were established from morphine-spiked plasma samples for different concentration ranges. The 3.05-m column used gave the greater separation of morphine and nalorphine peaks with less interferences at low morphine concentrations than the 1.5-m column used previously (28).

Radioisotopic Derivatization Method with ^{3}H-Dansyl Chloride—Plasma (100 μl) was placed in a 5-ml centrifuge tube containing 400 μl of the previously described 1 *M* carbonate buffer (pH 8.9). The mixture was mixed with 1 ml of benzene–1-butanol (85:15) on a vortex mixer for 30 sec. The phases were separated by centrifugation for 15 min, and the organic phase was transferred to a 1-ml vial[3] with a Pasteur pipet. The extraction was repeated with 1 ml of solvent, and the combined solvent extracts were evaporated under a nitrogen stream at 55° on a heating block.

The vial walls were washed with 100 μl of solvent, and the contents of the vial were taken to dryness. A 100-μl benzene solution of ^{3}H-dansyl chloride, which was diluted to about 0.83-Ci/mmole specific activity with unlabeled dansyl chloride, was added to the dry residue, and the benzene was evaporated under a nitrogen stream at room temperature. The dry residue, containing the mixture of dansyl chloride and morphine, was dissolved in 20 μl of acetone, and 5 μl of 0.1 *M* sodium bicarbonate was added. The mixture was reacted in a dark place for 7 hr at room temperature. Excess reagent was destroyed with 50 μl of 0.1 *M* sodium carbonate, and the dansylated morphine was extracted with 200 μl of chloroform–isopropyl alcohol (3:1).

After the separation of the phases by centrifugation, the aqueous phase was removed carefully with a 50-μl syringe and discarded. The organic solvent was evaporated under a nitrogen stream at room temperature for

[17] Reacti-Term heating module, Pierce Chemical Co., Rockford, Ill.
[18] Model 3380A integrator, Hewlett-Packard, Avondale, Pa.

Table I—Regression Analyses ($R = mC + b$) of Calibration Curves for GLC Morphine Assay: Peak Area or Height Ratio (R) [a] against Morphine Concentration (C, mg/ml) in Plasma on Analysis of 0.5 ml

C, Range, ng/ml	Internal Standard, ng/ml	m ($\pm \sigma_m$)	b ($\pm \sigma_b$)	σ_R [b]	C_1 [c], ng/ml	σ_c	SD, % of C_1
926–3705	2122	4.6 (±0.1) × 10^{-4}	1.27 (±0.04) × 10^{-2}	0.012	2500	26	1.0
371–936	510	1.70 (±0.10) × 10^{-3}	1.27 (±0.78) × 10^{-1}	0.059	500	34	6.8
18.5–92.6	56.6	1.06 (±0.04) × 10^{-2}	1.9 (±2.6) × 10^{-2}	0.025	50	2.4	4.8
1.8–9.2 [d]	5.65	1.24 (±0.11) × 10^{-1}	3.0 (±6.1) × 10^{-2}	0.058	5	0.46	9.2

[a] Peak height ratios were used only in the 1.8–9.2 ng/ml (Fig. 2). [b] From variance about regression. [c] Value of C_1 to substitute in regression equation for calculation of $\pm\sigma_c$ from given $\pm\sigma_R$. [d] Plasma, 1.0 ml, was extracted in this case.

approximately 20 min. The dry residue was dissolved in 20 μl of chloroform–isopropyl alcohol (3:1), and 10 μl of this solution was spotted on a silica gel plate. The plate was developed with methanol (Fig. 2).

The spot assigned to morphine dansylate was identified under UV light (365 nm), marked, scraped, and transferred to a scintillation vial. Methanol (0.5 ml) was added and then shaken with the gel for 5 min, and liquid scintillation fluid (10 ml) was added. The capped samples were dark adapted for at least 6 hr before counting. A calibration curve (Fig. 3) was constructed from spiked plasma samples carried through the derivatization and TLC procedures at the same time as the samples to be analyzed.

Radioactive Assay of ^{14}C-Morphine—Plasma or urine (0.5 ml) was placed in a 15-ml centrifuge tube containing 1.5 ml of 1 M carbonate buffer (pH 8.9) prepared as described previously. The mixture was extracted with 4 ml of ethyl acetate–isopropyl alcohol (85:15) by mixing for 30 sec on a vortex mixer, and the tubes were centrifuged for 10 min at 3000 rpm. Then a portion (3 ml) of the organic phase was transferred into a liquid scintillation vial, and the residual organic phase was removed by aspiration. The aqueous phase was saved for subsequent assay of morphine conjugate.

The organic phase in the vial was evaporated under a nitrogen stream at 55° in a water bath. The dry residue was dissolved in 0.5 ml of methanol, 10 ml of liquid scintillation fluid was added, and the samples were dark adapted for at least 6 hr before counting. All samples also were counted after addition of a known amount of the internal standard, ^{14}C-urea (usually 50,000 dpm), to calculate the counting efficiency. The counts per minute values were converted to disintegrations per minute by dividing by the counting efficiency after subtracting background.

Total Radioactivity—The total radioactivity of a plasma or urine sample was determined from an aliquot (0.2 or 0.5 ml) of plasma or urine transferred to a liquid scintillation vial with 0.5 ml of water added. Liquid scintillation fluid (10 ml) was added to the vial, and the capped samples were dark adapted for at least 6 hr. The counting efficiency was calculated by the internal standard method already described. The total concentration of radiolabeled substances as morphine was calculated by dividing the disintegrations per minute values by the known specific activity of the morphine.

^{14}C-Morphine Glucuronide—Morphine glucuronide was hydrolyzed by the procedure of Yeh and Woods (15). The previously morphine-extracted and diluted plasma and urine samples (0.5 ml) were adjusted to about 3.2 M HCl with 4 ml of 6 M HCl. The capped tubes were maintained for 30 min at 4.54-kg pressure in an autoclave, and the hydrolyzed samples were carefully adjusted to pH 8.9 with solid sodium carbonate.

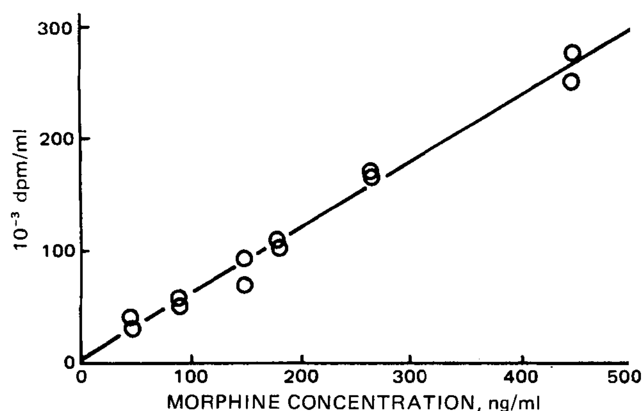

Figure 3—*Calibration curve for morphine derivatized with ^{3}H-dansyl chloride after extraction from 100 μl of plasma.*

The free morphine was extracted by the techniques previously described, and the content of the organic solvent was analyzed by liquid scintillation counting for morphine derived from its conjugates. For GLC, the organic extract was extracted by 3 ml of hydrochloric acid, and 1 ml of the hydrochloric acid was assayed by the procedures used in GLC monitoring.

Preparation of Morphine Dansylate—Crystalline morphine sulfate (0.125 mM) was dissolved in 2 ml of water, and 2 ml of the acetone solution of dansyl chloride (0.250 mM) and 1 g of sodium bicarbonate were added. The mixture was reacted for 2 hr at room temperature. The yellow precipitate was filtered, washed with water, and recrystallized from acetone–water. The filtrate was extracted with ethyl acetate. This procedure gave additional morphine dansylate, and the overall yield was 49%. The mass spectrum of the product was taken after TLC purification and showed the molecular peak at m/e 518 for monodansylated morphine (Fig. 4).

Determination of Extraction Efficiency of Morphine from Plasma—The extraction efficiency of a known amount of TLC-purified ^{14}C-morphine added to dog plasma (129 ng/ml) was determined using the method described under *Radioactive Assay of ^{14}C-Morphine* and was 97.3 ± 0.9% (*SD*).

Preparation of Pharmacokinetic Dose—N-Methyl-labeled ^{14}C-morphine hydrochloride[5], 60 mCi/mmole (1.197 mg as morphine base), was mixed with 20 mg of crystalline morphine sulfate (15.039 mg as morphine base) to give a theoretical specific activity of morphine of 4.39 μCi/mmole. The mixture was dissolved in 3.5 ml of sterile isotonic saline, and 3 ml was injected into the dog. The total dose was 13.91 mg as morphine base. The actual specific activity of the dose was determined by HPLC.

The N-methyl-labeled ^{14}C-morphine was spotted on a silica gel plate and developed with methanol–ammonia (99:1). Each centimeter of the plate was scraped, eluted with 0.5 ml of methanol, and counted after the addition of 10 ml of liquid scintillation fluid; 87% of the radioactivity was found at the morphine spot (R_f 0.43). This same method was used to prepare the purified ^{14}C-morphine used in studies of extraction efficiencies.

Aliquots of the prepared dose (5 μl) were analyzed for radiolabeled purity by HPLC with a reversed-phase column, using 45% methanol–0.0002 M dibasic sodium phosphate (adjusted to pH 7.8 with 0.1 M HCl) at a constant flow rate of 2 ml/min. The eluate was monitored for UV absorbance at 254 nm and collected in a scintillation vial over the range (6–7.5 min) where the retention volume of morphine was 6.7 min. The collected fraction was taken to dryness under nitrogen at 70° and dissolved in 0.5 ml of methanol.

Liquid scintillation fluid (10 ml) was added, and the pure radiolabeled morphine was counted after 6 hr of dark adaptation. The amount of

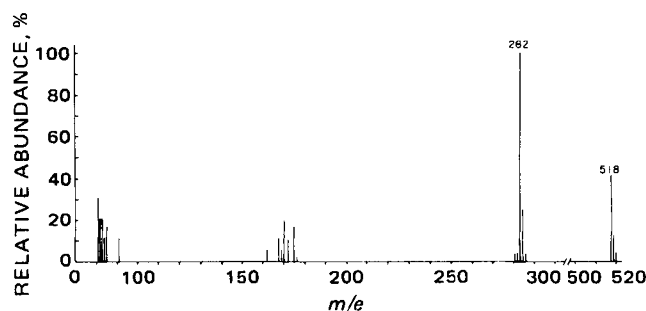

Figure 4—*Mass spectrum of morphine dansylate to demonstrate that the derivatization of morphine with cold dansyl chloride occurs at only one hydroxyl with a resultant product of mass* m/e *518.*

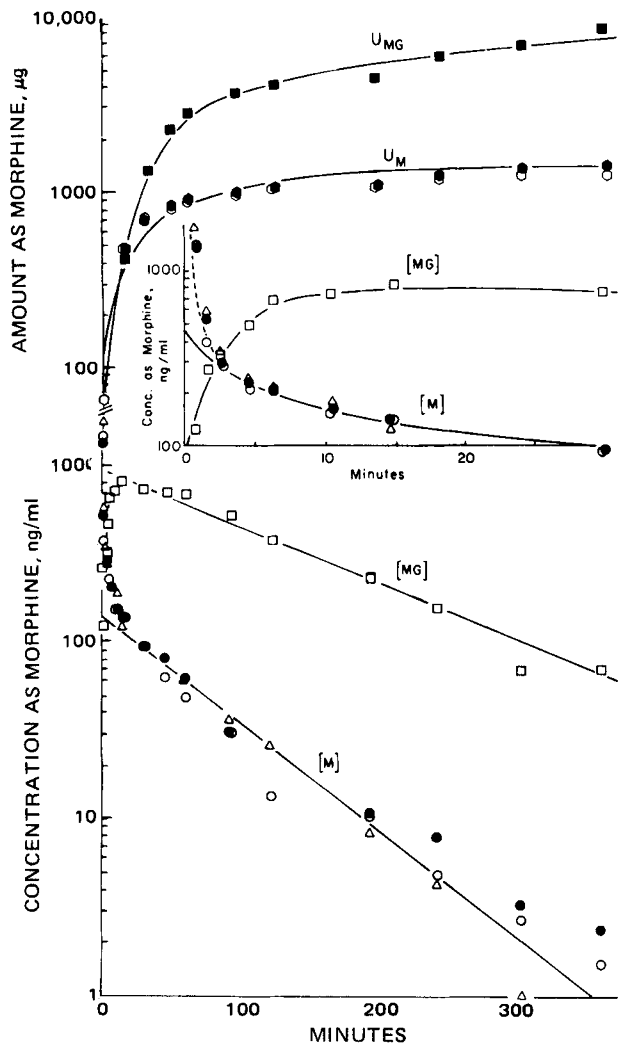

Figure 5—*Plots against time of morphine concentrations in plasma, [M], assayed by liquid scintillation spectrophotometry* (O) *and GLC* (●) *of the hexane extract of 0.5 ml of plasma and by dansyl chloride derivatization* (△) *of the hexane extract of 0.1 ml of plasma. The plot against time of morphine glucuronide, [MG], was based on the average of the values obtained from the total liquid scintillation counting of 0.2 ml of plasma and the counting of the hexane extract of 0.5 ml of solvolyzed plasma from which the morphine had been removed by extraction prior to solvolysis. The cumulative plots against time of morphine,* U_M (O), *and it glucuronide,* U_{MG} (□), *excreted in the urine were based on liquid scintillation counting of urine before and after hexane extraction. The values,* U_M (●), *based on the GLC assay of the hexane extract of urine are given also. The lines drawn through the [M] and [MG] data are those of best fit. The curve drawn through the* U_{MG} *data was generated from* $U_M = Cl_M^{ren} \int_0^t [M]\, dt$, *where* Cl_M^{ren} *was taken as 120 ml/min and* $\int_0^t [M]\, dt$ *is the area under the [M] versus time plot up to time* t. *The curve drawn through the* U_{MG} *data was generated from* $U_{MG} = Cl_{MG}^{ren} \int_0^t [MG]\, dt$, *where* Cl_{MG}^{ren} *was taken as 60 ml/min and* $\int_0^t [MG]$ dt *is the area under the [MG] versus time plot up to time* t. *The inset is for [M] and [MG] on an expanded time scale. The solid line through [M] is for calculated values based on a sum of two exponentials, which ignores the two points at the earliest times; the dashed line in the inset is based on a sum of three exponentials.*

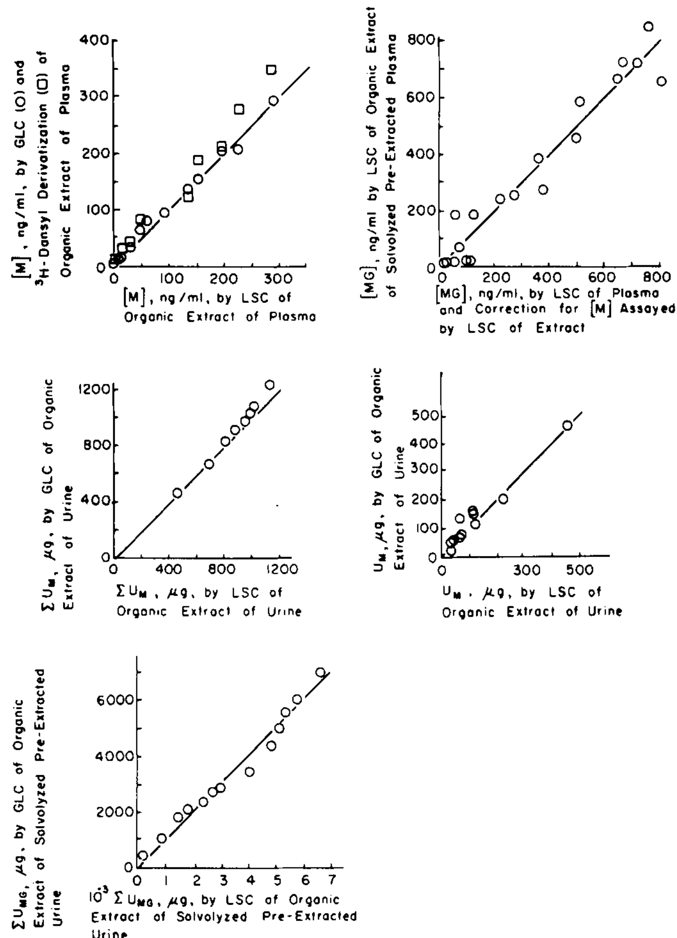

Figure 6—*Regression plots of one assay upon another for the various analytical methods used to determine concentrations of morphine, [M], and morphine conjugates, [MG], in plasma; amounts of morphine,* U_M, *in urine collections; and cumulative amounts of morphine,* ΣU_M, *and conjugate,* ΣU_{MG}, *in the urine of a dog administered a 14.00-mg iv dose of morphine. The theoretical lines drawn through the points have slopes of unity and intercepts of zero. LSC = liquid scintillation counting.*

morphine in the collection was determined with respect to a prior HPLC calibration of the UV peak heights on HPLC with pure cold morphine. Thus, the true specific activity was 29,993 dpm/µg or 3.85 µCi/mmole, and 4.216×10^8 dpm (14.0 mg) was administered to the 15.2-kg dog.

Treatment of the Animal—The dog was transferred to a metabolism cage 1 day before the experiment. It was weighed and anesthetized with pentobarbital (30 mg/kg iv). One external jugular vein of the neck was exposed and cannulated under sterile conditions with 30 cm of a catheter[19], and at least 20 cm was inserted into the vein. The incision was closed after the patency of the cannula was verified, and it was filled with heparinized saline. On the following day, the dog was placed on a table in a standing position and restrained by two straps around the fore- and hindlegs that were fixed to a horizontal bar above.

^{14}C-Morphine solution was prepared in isotonic sterile saline and injected into the jugular catheter over 20 sec. The dosing syringe and catheter were flushed with 10 ml of normal saline. Blood was withdrawn from the jugular catheter, after the dead space of the catheter was filled with fresh undiluted blood, into a sterile, disposable syringe containing 0.01 ml of heparin (10,000 µl/min). The blood sample was transferred to a 5-ml centrifuge tube and centrifuged at 1500 rpm for 10 min. Plasma was transferred to another 5-ml centrifuge tube with a Pasteur pipet, and the tube was stoppered and refrigerated.

Blood (5 ml) was sampled at 0.5, 1, 2, 4, 6, 10, 15, 30, 45, 60, 90, 120, 190, 240, 300, 360, 420, 480, 540, 625, and 1435 min. Hematocrits were obtained on selected blood samples prior to centrifugation.

Urine was collected from the catheterized animal at approximately 15, 30, 50, 60, 90, 120, and 190 min and then every 60 min up to 420 min, every 120 min up to 640 min, and every 12 hr up to 2 days. The volumes of each collection were measured. Urine pH was determined, and an aliquot was removed to determine total carbon-14. The remaining urine was stored in a refrigerator.

The dog was returned to the metabolism cage 12 hr after drug administration.

[19] Medical grade tubing, Dow Corning Corp., Medical Products, Midland, MI 48640.

Table II—Comparisons of Various Assays of Biological Fluids with Time by Regression Analyses [$y \pm \sigma_y = m(\pm\sigma_m)x + b(\pm\sigma_b)$] of Concentrations of Morphine, M, and Morphine Conjugates, MG, in Plasma ([M] and [MG]), Amounts in a Urine Collection (U_M and U_{MG}), and Amounts Cumulatively Excreted in Urine (ΣU_M and ΣU_{MG}) after a Morphine Dose of 14.00 mg to a Dog

Assay	y	x	n	Range	$m \pm \sigma_m$	$b \pm \sigma_b$	σ_y	r^2
[M], ng/ml	GLC assay of organic extract of 0.5 ml of plasma	LSC[a] assay of organic extract of 0.5 ml of plasma	6	90–300	0.986 ± 0.070	1.4 ± 14	10.8	0.981
			10	10–300	0.956 ± 0.035	7.9 ± 5.4	9.8	0.989
			12	10–1400	0.970 ± 0.036	17 ± 16	45	0.987
			18	0.2–1400	0.979 ± 0.027	10 ± 9.7	36	0.988
	^{3}H-Dansyl derivatization of organic extract of 0.1 ml of plasma	LSC assay of organic extract of 0.5 ml of plasma	10	4–350	1.167 ± 0.057	−1.7 ± 8.5	18	0.981
			12	4–1811	1.31 ± 0.03	−10 ± 11	32	0.996
[MG], ng/ml	LSC of 4 or 5 of 12 ml of organic extract of solvolyzed preextracted 0.5 ml of plasma	Determination from LSC (total counts) of 0.1 ml of plasma and LSC of organic extract of 0.5 ml of plasma	11	200–900	0.992 ± 0.132	−1.3 ± 75	85	0.992
			20	14–900	0.992 ± 0.060	−1.9 ± 25	74	0.938
U_M, μg	GLC assay of organic extract of 0.5 ml of urine	LSC assay of organic extract of 0.5 ml of urine	5	60–500	0.960 ± 0.098	12 ± 24	33	0.970
			13	20–500	0.938 ± 0.065	22 ± 10	27	0.950
ΣU_M, μg	GLC assay of organic extract of 0.5 ml of urine	LSC assay of organic extract of 0.5 ml of urine	7	0–1100	1.11 ± 0.05	−72 ± 40	23	0.991
			13	0–1625	1.23 ± 0.05	−213 ± 47	41	0.988
ΣU_{MG}, μg	GLC assay of organic extract of 0.5 or 1.0 ml of 3 ml of aqueous solution from material in organic extract of solvolyzed preextracted 1.0 ml of urine	LSC of 5 of 12 ml of organic extract of solvolyzed preextracted 0.5 ml of urine	13	0–7000	0.963 ± 0.045	180 ± 177	320	0.977

[a] LSC = liquid scintillation counting.

RESULTS AND DISCUSSION

GLC Assay of Plasma Morphine—The regression analyses of linear calibration curves for plasma morphine obtained in four different concentration ranges are given in Table I. In three of the four cases, the intercepts were not significantly different from zero and the estimated standard deviations (in percent of concentration) of an assay from 0.5 ml of plasma ranged from 1% at 2500 ng/ml to 9.2% at 5 ng/ml.

Radioisotopic Derivatization Method for Morphine—Loh *et al.* (21) derivatized morphine extracted from biological samples with dansyl chloride and separated the dansylated morphine from reagent and other reaction products by TLC on polyamide plates. The detection of dansyl morphine was effected by fluorescent scanning or by radioactive counting when ^{14}C-morphine had been used. The sensitivity was claimed to be 5 ng.

The radioisotopic derivatization of morphine described herein was based on the reaction of ^{3}H-dansyl chloride with nonlabeled morphine after a single-step extraction from plasma. The facile TLC separation was applied with subsequent liquid scintillation counting of the ^{3}H-dansylated morphine. This procedure substituted for the extensive sequence of solvent extractions necessary for the cleanup prior to GLC assay of derivatized morphine. The radioisotopic derivatization assay of nonradiolabeled morphine in plasma demonstrated a linear calibration curve (Fig. 3) with a regression equation for the specific activity of ^{3}H-dansyl chloride used (0.83 Ci/mmole) of:

$$\text{dpm/ml} (\pm 11 \times 10^3) = 592 (\pm 25)C + 3.0(\pm 5) \times 10^3 \quad \text{(Eq. 1)}$$

for a concentration, C, in nanograms of morphine per milliliter of plasma. The parenthetical values are the standard deviations. The first two parentheses are related to the variance about regression and the variance of the slope. The intercept is not significantly different from zero. The error about regression permitted an estimation of a concentration, C, from a given monitored disintegrations per minute of ±18 ng/ml when 0.1 ml of plasma was taken. The sensitivity could be increased to ±2 ng when 1 ml of plasma was taken. Calibration curves should be prepared on the day of assay since they demonstrated variation among days.

Comparisons of Assays—The plasma samples from a 15-kg dog injected with 14 mg of ^{14}C-morphine (30,000 dpm/μg) through a jugular catheter were analyzed for morphine concentrations, [M], as a function of time by the electron-capture GLC assay, the radioactive dansyl derivatization assay, and the direct radiochemical assay after selective extraction (Fig. 5). Linear relationships were found for both the GLC and radioactive dansyl derivatization assays with the direct radiochemical assay of the organic solvent extract for morphine (Fig. 6). The regression coefficients, m, were not significantly different than unity, nor were the intercepts significantly different than zero (Table II). There were no significant differences by the t test between the mean values of these methods.

The concentrations of morphine conjugates in plasma, [MG], were assayed with time (Fig. 5). There was a linear relation with a slope not significantly different than unity and with an intercept not significantly different than zero for the regression of morphine conjugate concentration by liquid scintillation counting of the organic extract of acid-hydrolyzed plasma, which had been preextracted to remove unconjugated morphine, and the conjugate assayed from the difference between total radioactive counts of plasma and the samples extracted as morphine in the organic solvent (Fig. 6 and Table II).

Similarly, the time courses of amounts of morphine excreted in the urine, U_M, by the GLC assay and radiolabeled counting of the organic extracts of urine were the same (Fig. 5). The regression coefficients of the values of the former on the latter (Fig. 6) were not significantly different than unity with an intercept of zero. Furthermore, the equivalence of methods for morphine conjugates in urine, U_{MG}, was demonstrated. GLC assay of the organic extract of solvolyzed preextracted urine plotted against the radiolabeled counting of the organic extract gave a slope of unity and an intercept of zero (Fig. 6 and Table II).

Initial Pharmacokinetic Study—The time course of morphine in the plasma of the 15.2-kg dog administered 14.0 mg iv of morphine (Fig. 5) demonstrated a dependency on the sum of three exponentials. If the initial two values taken at 0.75 and 1.6 min were ignored, a sum of two exponentials was adequate with respective apparent half-lives of 3.0 and 48.8 min. If the initial two points were accepted, an additional apparent half-life of 0.23 min was indicated. The calculated pharmacokinetic parameters for this study are listed in Table III for both models, and their methods of calculation are given in the footnotes.

On the premise of the two-compartment body model, the apparent volume of distribution of the central compartment for morphine was 31 liters; it was 1.3 liters for the three-compartment body model. The latter value is close to the plasma volume of a 15-kg dog (0.6–1.2 liters) whereas the former greatly exceeds the total body water (9–12 liters) (36). These facts strongly indicate that an α'-phase does exist. This finding is confirmed by achievement of three-fourths of the maximum in morphine conjugate concentration in the plasma within 5 min (Fig. 5) after administration when the renal clearance of this conjugate is a relatively slow process (58 ml/min).

The hepatic flow in the 15-kg dog is in the 900–1300-ml/min range (37), which corresponds well to the estimated metabolic clearance of morphine of 959–1234 ml/min. This result indicates complete metabolism of all morphine entering the liver, and an extremely high first-pass metabolism on oral administration would be anticipated. The 86–120-ml/min esti-

Table III—Pharmacokinetic Parameters on Intravenous Bolus Administration of 14.0 mg of Morphine to a 15.2-kg Dog

Body Model[a]	Two Compartment	Three Compartment
A', ng/ml	0	10,000
A	310	310
B	142	142
α', min^{-1}	∞	307
α	0.230	0.230
β	1.42×10^{-2}	1.42×10^{-2}
C_0, ng/ml[b]	452	10,452
$V_C{}^M$, ml[c]	30,973	1339
Cl^M_{ren}, ml/min[d]	120.0 ± 9.9 (*SD*)	86.2 ± 5.2
Cl^{MG}_{ren}, ml/min[e]	57.9 ± 5.5 (*SD*)	57.9 ± 5.5 (*SD*)
Cl^M_{tot}, ml/min[f]	1234	959
Cl^M_{met}, ml/min[g]	1176	901
Fraction of dose renally excreted as *MG*	0.643	0.643
Fraction of dose renally excreted as *M*[h]	0.102	0.102
$Cl^{M \rightarrow MG}$, ml/min[i]	840	840
$k_{[MG],U_{MG}}$, min^{-1} [j]	7.09×10^{-3}	7.09×10^{-3}
V^{MG}_{pss}, ml[k]	8166	8166
V^M_{pss}, ml[l]	86,900	67,535

[a] The time course of concentration of morphine in plasma can be described by $[M] = A'e^{-\alpha' t} + Ae^{-\alpha t} + Be^{-\beta t}$. [b] $C_0 = A' + A + B$. [c] Volume of central compartment referenced to morphine concentration in plasma, $V_C{}^M = D/C_0$, where $D = 14 \times 10^6$ ng. [d] Renal clearance of morphine was calculated from the average of values, $Cl^M_{ren} = \Sigma U_M / \int_0^t [M]\, dt$, where ΣU_M is amount of morphine excreted in the urine up to time t obtained by interpolation of the ΣU_M *versus* t curve after 15 min and $\int_0^t [M]\, dt$ is the area under the $[M]$ *versus* t curve up to time t, $n = 13$. [e] Renal clearance of morphine conjugate calculated similarly where $Cl^{MG}_{ren} = \Sigma U_{MG} / \int_0^t [MG]\, dt$. [f] Total clearance of morphine $Cl^M_{tot} = D / \int_0^\infty [M]\, dt$, where $\int_0^\infty [M]\, dt$ is the total area under the $[M]$ *versus* time curve and can be calculated from $A'/\alpha' + A/\alpha + B/\beta$. [g] $Cl^M_{tot} - Cl^M_{ren}$. [h] Value by liquid scintillation counting where value by GLC was 0.116. [i] Clearance of morphine to analyzed conjugate estimated from fCl^M_{met}, where $f = \Sigma U_{MG}/(D - \Sigma U_M) = 0.71$. [j] Estimated from terminal slope of ln $[MG]$ *versus* time. [k] Pseudo-steady-state volume of distribution estimated from $Cl^{MG}_{ren}/k_{[MG],U_{MG}}$. [l] Pseudo-steady-state volume of distribution estimated from Cl^M_{tot}/β.

mates of renal clearance of morphine and the 58-ml/min clearance of the conjugates are well within the 40–130-ml/min range assigned to glomerular filtration by inulin clearance (36). The estimates of the renal clearances are consistent with the observed excretions of morphine and its conjugates in the urine (Fig. 5).

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