(3) Y. Gibor, S. Bhuvarasarma, and A. Scommegna, *ibid.*, **22**, 671 (1971).
(4) B. D. Kulkani, T. D. Avila, B. B. Pharriss, and A. Scommegna, *Contraception*, **8**, 299 (1973).
(5) A. Scommegna, T. Avila, M. Luna, R. Rao, and W. P. Dmowski, *Obstet. Gynecol.*, **43**, 769 (1974).
(6) S. A. Tillson, M. Marion, R. Hudson, P. Wong, B. B. Pharriss, R. Aznar, and J. Martinez-Manautou, *Contraception*, **11**, 179 (1975).
(7) B. B. Pharriss, R. Erickson, J. Bashaw, S. Hoff, V. A. Place, and A. Zaffaroni, *Fertil. Steril.*, **25**, 915 (1974).
(8) W. P. Dmowski, A. Shih, J. Wilhelm, F. Auletta, and A. Scommegna, *ibid.*, **28**, 262 (1977).
(9) H. J. Tatum, *Am. J. Gynecol.*, **112**, 1000 (1972).
(10) R. W. Baker and H. K. Lonsdale, in "Controlled Release of Biologically Active Agents," A. C. Tanquary and R. E. Lacey, Eds., Plenum, New York, N.Y., 1974.
(11) J. W. Boretos, D. E. Detmer, and J. H. Donachy, *J. Biomed. Mater. Res.*, **5**, 373 (1971).
(12) H. K. Lonsdale, U. Merten, and R. L. Riley, *J. Appl. Polym. Sci.*, **9**, 1341 (1965).
(13) R. L. Shippy, S. T. Hwang, and R. G. Bunge, *J. Biomed. Mater. Res.*, **7**, 115 (1973).
(14) J. Crank, "The Mathematics of Diffusion," Oxford University Press, Oxford, England, 1967.
(15) K. Lehmann, *Drugs Made Ger.*, **10**, 115 (1967).
(16) C. A. Noll and L. J. LaSalvia, U.S. pat. 3,468,796 (Sept. 23, 1969).

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Pharmacokinetics of Morphine and Its Surrogates II: Methods of Separation of Stabilized Heroin and Its Metabolites from Hydrolyzing Biological Fluids and Applications to Protein Binding and Red Blood Cell Partition Studies

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Abstract □ The inhibition of the spontaneous hydrolysis of heroin in fresh dog plasma and blood ($t_{1/2}$ = 8 min) is effected by 10 mg of sodium fluoride/ml ($t_{1/2}$ = 40 min) and 35 μg of tetraethyl pyrophosphate/ml ($t_{1/2}$ = 415 min). Tetraethyl pyrophosphate is the inhibitor of choice and gives the same stability for heroin as in phosphate buffer. Aged plasma loses its enzymatic efficiency. Heroin in cerebrospinal fluid hydrolyzes at rates similar to those in buffer. Modified extraction procedures developed for enzyme-inhibited plasma at pH 4.5 have high extraction efficiencies (86–100%) and permit isolation of undegraded heroin from its metabolites. Separations of heroin and metabolites from enzyme-inhibited plasma were effected by described high-pressure liquid chromatographic systems and from TLC with elution of pertinent developed spots. Efficiencies of these TLC recoveries were 81 ± 1% for heroin and 82 ± 1% for morphine. Contrary to the literature, heroin has significant protein binding where 40% of that not bound to an ultrafiltration membrane is bound to dog plasma proteins. The apparent partition coefficient is 1.4 ± 0.2 between red blood cells and plasma water, and it is 0.8 ± 0.1 between red blood cells and dog plasma.

Keyphrases □ Heroin—hydrolysis in dog plasma and blood, effect of sodium fluoride and tetraethyl pyrophosphate, protein binding and partition coefficient evaluated □ Hydrolysis—heroin in dog plasma and blood, effect of sodium fluoride and tetraethyl pyrophosphate □ Enzyme inhibition—by sodium fluoride and tetraethyl pyrophosphate, dog plasma and blood, effect on heroin hydrolysis □ Protein binding—heroin in enzyme-inhibited dog plasma □ Partition coefficient—heroin between red blood cells and enzyme-inhibited dog plasma □ Narcotics—heroin, hydrolysis in dog plasma and blood, effect of enzyme inhibitors, protein binding and partition coefficient evaluated

3,6-Diacetylmorphine (heroin) is rapidly hydrolyzed in the body to 6-monoacetylmorphine and then to morphine (1–3). These latter compounds, along with morphine 3-glucuronide, are the major metabolites of heroin excreted in urine (4–8), although minor or negligible amounts of normorphine and its glucuronide as well as morphine 6-glucuronide and 6-acetylmorphine were reported in human urine after heroin administration (9–11).

BACKGROUND

Other than the heroin assay of Way *et al.* (2, 3) on homogenized mice after intravenous administration of heroin ($t_{1/2}$ for heroin = ~2.5 min), the inability to monitor heroin levels in plasma or biological fluid due to its fast hydrolysis and/or metabolism has prevented the study of heroin pharmacokinetics *per se*. Pharmacokinetic studies on heroin administration were thus limited to the estimation of half-lives of urinary excretions of the major heroin metabolites with apparent $t_{1/2}$ values of 1–3 hr (8, 10).

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The apparent fast metabolism of heroin *in vivo* is confounded further by its rapid *in vitro* hydrolysis in aqueous solutions and biological fluids (12). The limited studies available showed that heroin hydrolyzes to 6-*O*-acetylmorphine in 0.5 *M* sodium carbonate with a half-life of 4.2 min whereas the subsequent hydrolysis to morphine has a half-life of 55.5 min (12). Human blood was reported to be devoid of 6-monoacetylmorphine esterase, although the rate of morphine liberation was slightly faster than in nontreated serum (13). Heroin was stable at pH 7.3 in phosphate buffer at 37.5° (12) but not in enzyme-deactivated human serum (13).

Hydrolysis in whole human blood ($t_{1/2}$ = 12.6 min) was faster than the hydrolysis in serum ($t_{1/2}$ = 19.8 min). Nakamura *et al.* (12) also implied that 1% sodium fluoride might inhibit *post-mortem* hydrolysis of, or bacterial action on, heroin in plasma.

These heroin instabilities in aqueous solutions and biological fluids cast suspicion on the validity of stated heroin quantifications in such fluids when they are not qualified for stability or when biological tissue treatment has used pH adjustment to 8 or 9 prior to the presumed extraction of heroin. Thus, the one available study of the heroin time course by analysis of homogenized organs and mouse tissue is also suspect (3). Furthermore, the inability to assay heroin after adjustment of blood and urine to pH 8.5–8.7 with saturated sodium bicarbonate prior to the presumed extraction (8) may be due to heroin degradation during the analytical processes as well as to its fast transformation *in vivo*. Tissue treatment with 10% trichloroacetic acid, with homogenization prior to subsequent extraction of the neutralized suspension (14), should give invalid estimates of easily hydrolyzable heroin and its products of acid solvolysis.

This paper presents methods of extraction and separation, such as high-pressure liquid chromatography (HPLC) and TLC, of heroin and its metabolites from biological fluids that minimize their spontaneous hydrolysis and permit quantitative assays. The efficiencies of various processes are given.

Sodium fluoride (12) and tetraethyl pyrophosphate (13) might inhibit the enzymic hydrolysis of heroin in plasma. The latter may be a preferred inhibitor since it was effective against a wide spectrum of esterases. The studies described here were designed to determine which inhibitor was the best to add to plasma prior to extraction under conditions where spontaneous hydrolysis would be minimized.

The only previous studies (15) to determine the protein and red blood cell binding of heroin were conducted by equilibrium dialyses over 18 hr with human serum. No consideration apparently was given to the possible hydrolysis of heroin in such systems, and the protein binding analysis was based on the assumption that the measured radioactivity in the compartments after equilibration would serve as a basis for calculation. But the results were contradictory and confusing. ^{14}C-Heroin showed no significant binding, but significant binding (20–39%) was observed when cold heroin was added to human serum. Similar results were obtained with washed human erythrocytes and red cell ghosts. It was concluded that "free heroin and morphine probably float unbound in the blood before . . . metabolism."

The probable instability of heroin under the experimental conditions of equilibrium dialysis of serum (15) demands a faster method to study protein binding, with an enzymic inhibitor to prevent heroin solvolysis. This paper considers the protein binding and red blood cell partition of heroin when these factors have been taken into account.

EXPERIMENTAL

Materials—*N-Methyl*-^{14}C-morphine[1], morphine sulfate crystalline USP[2], 6-*O*-acetylmorphine[3], and tetraethyl pyrophosphate[4] were used. All solvents were analytical grade[5]. Precoated silica gel plates[6], preparative and analytical, were used for TLC separation. The pH of the isotonic phosphate buffer was 7.45 (5.15 g of sodium chloride[5], 132 ml of 0.067 *M* KH_2PO_4[5], and *q.s.* to 1000 ml with 0.067 *M* $Na_2HPO_4 \cdot 7H_2O$[5]).

Freshly prepared 1 *M* $NaHCO_3$[5] was adjusted to pH 8.9 with 1 *M* NaOH[5]. Sodium acetate[5], 1 *M*, was adjusted to pH 4.8 with 1 *M* acetic acid[5]. Sodium carbonate[5], sodium fluoride[5], acetic anhydride[5], and pyridine[5] also were used.

Special high quality methanol[7] was used for HPLC separation. Ultrafiltration cones[8], cone supports[8], and tubes[8] were used for protein binding studies. Iodoplatinate spray was prepared from platinum chloride[5], according to the procedure of Stahl (16).

A toluene base scintillation fluid[9] was used for radioactive counting with a liquid scintillation counter[10]. All samples were counted for 5 min, with a blank inserted at the start. The degree of quench of each sample was estimated by the channel ratio method and based on an established quench curve. All samples were allowed to adapt to the dark for at least 6 hr. The systems and procedures used gave counting efficiencies of 95% when plasma was assayed and 92% when urine was assayed.

The chromatograph[11] was equipped with a 421.8-kg/cm^2 (6000-psi) constant flow pump and a 254-nm UV detector. A low polarity reversed-phase column[12] was used.

All glassware was silylated[13].

Preparation of ^{14}C-Heroin—The ^{14}C-heroin was synthesized by acetylation of ^{14}C-morphine (59 mCi/mmole). ^{14}C-Morphine was mixed with unlabeled crystalline morphine sulfate to obtain a theoretical 10–15 mCi/mmole specific activity. The mixture (10–15 mg as morphine base) was acetylated with acetic anhydride (0.5 ml) in the presence of pyridine (5–10 μl) for 3 hr in a tightly capped 15-ml test tube submerged in a 70° water bath[14] and was maintained at room temperature for 2 days. Excess acetic anhydride was evaporated under a nitrogen stream at 50°, and 2 ml of 5% sodium carbonate solution was added to the brown–pinkish residue.

The heroin was extracted from the aqueous solution five or six times with 5 ml of chloroform or until a small amount of chloroform solution spotted on a TLC plate did not give a positive reaction with iodoplatinate spray. The combined chloroform phases were evaporated to 1 ml under a nitrogen stream at 50° in a water bath. This solution was applied to a preparative TLC (silica gel) plate, and the plate was developed with chloroform–methanol (80:20). The heroin band (R_f 0.77) was scraped from the plate and extracted several times with 20 ml of acetone until the TLC spotted extracts showed no positive reaction with iodoplatinate spray.

The combined acetone phases were evaporated under a nitrogen stream on a heating block[15] at 55°. The obtained heroin was of high purity (97.5%); only small amounts of radioactivity were obtained on the 6-*O*-acetylmorphine (2.24%, R_f 0.50) and morphine (0.12%, R_f 0.22) spots.

Nonlabeled heroin was prepared according to the procedure of Hays *et al.* (17).

Analysis of ^{14}C-Radiolabeled Heroin, 6-*O*-Acetylmorphine, and Morphine in Plasma—The procedure was used to determine the products of plasma solvolysis of ^{14}C-heroin. Tetraethyl pyrophosphate (10 μl) in an acetone solution of 17.3 mg/ml was added to a vial, and the acetone was evaporated. Plasma containing heroin (1.0 ml) was added to the vial, and the pH was adjusted to 4.8 with 1 ml of 1 *M* acetate buffer of the same pH. It was extracted immediately with 22 ml of benzene–butanol (85:15).

The organic phase (20 ml) was transferred to a scintillation vial and evaporated under a nitrogen stream in a water bath at 60°. The dry residue was dissolved in 0.5 ml of methanol, and 10 ml of scintillation fluid was added. The capped vials were dark adapted for at least 6 hr before counting. This procedure extracts heroin into the organic phase, and the remaining aqueous solution contains 6-*O*-acetylmorphine and morphine.

Carbonate buffer (2 ml) adjusted to pH 8.9 was added to the aqueous solution (pH 8.9), and 6-*O*-acetylmorphine and morphine were extracted with 2 × 5 ml of ethyl acetate–isopropyl alcohol (85:15). The combined organic phases in a 15-ml centrifuge tube were evaporated under a nitrogen stream in a water bath at 60°. The walls were rinsed with 0.5 ml of the same solvent. The solution was again evaporated to dryness. The dry residue was then dissolved in 25 μl of the same solvent with a vortex mixer, 15 μl was spotted on a silica gel TLC plate, and the plate was developed with chloroform–methanol (80:20).

6-*O*-Acetylmorphine and morphine standards were spotted on the same plate to determine their positions. The standards were visualized

[1] Amersham/Searle Co., Arlington Heights, Ill.
[2] Merck and Co., Rahway, N.J.
[3] Batch 1934-886-50, Department of Health, Education, and Welfare, Public Health Service, National Institute of Mental Education.
[4] Chemical Procurement Laboratories, College Point, N.Y.
[5] Mallinckrodt Chemical Works, St. Louis, Mo.
[6] Silica gel 60, E. Merck Laboratories, Elmsford, N.Y.
[7] Burdick and Jackson Laboratories, Muskegon, Mich.
[8] Type 2100 CF-50, Amicon Corp., Lexington, Mass.
[9] Scinti Verse, Fisher Scientific Co., Fair Lawn, N.J.
[10] Model LS 330, Beckman Instruments, Fullerton, Calif.
[11] Model ALC 202, Waters Associates, Milford, Mass.
[12] μBondapak C_{18} Porasil, Waters Associates, Milford, Mass.
[13] Silyl-8, Pierce Chemical Co., Rockford, Ill.
[14] Meyer N-Evap, Organomation Associates, Shrewsbury, Mass.
[15] Reacti-Therm heating module, Pierce Chemical Co., Rockford, Ill.

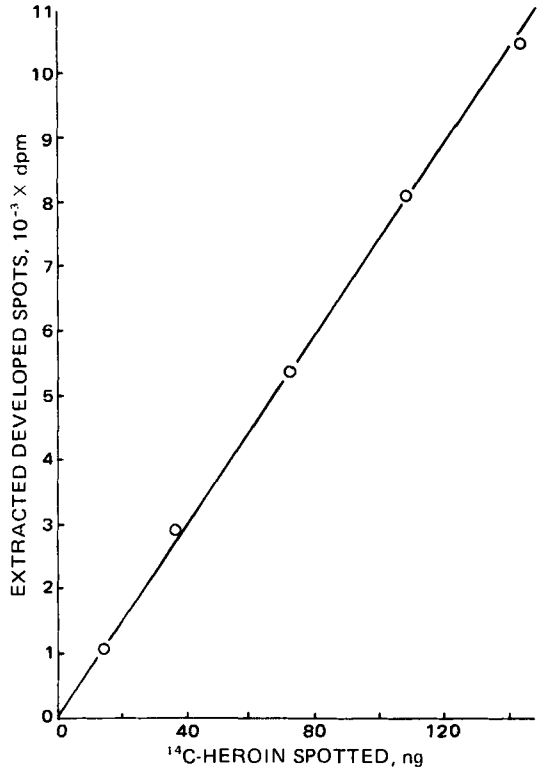

Figure 1—*Plot of disintegrations per minute of heroin extracted with 0.5 ml of methanol from spots at appropriate* R_f *values (0.77) on a TLC silica gel plate developed with chloroform–methanol (80:20) against the nanograms of* ^{14}*C-heroin spotted in 10 μl of acetone at the origin.*

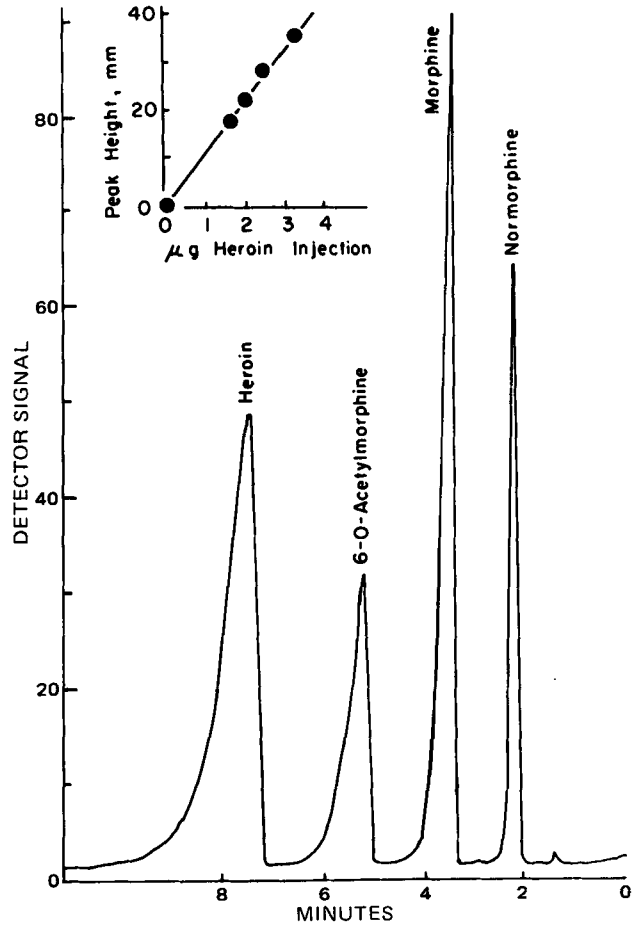

Figure 2—*HPLC reversed-phase chromatogram of a 10-μl aqueous solution of a mixture of heroin, 6-O-acetylmorphine, morphine, and normorphine developed by 55:45 methanol–buffer (2 ml/min) where the buffer contained 0.1% $(NH_4)_2CO_3$ and 0.01 M $(NH_4)_2HPO_4$. The inset is a typical calibration curve in millimeters of height of the heroin peak against the nanograms of heroin injected.*

with iodoplatinate spray. The marked spots were scraped from the plate, and the powdered silica was transferred to a vial and agitated with 0.5 ml of methanol for 5 min. Scintillation liquid (10 ml) was added, and the capped vials were counted after 6 hr of dark adaptation.

Hydrolysis Kinetics of Heroin in Body Fluids—The hydrolysis kinetics of heroin were studied in buffer, plasma, whole blood, and cerebrospinal fluid. The kinetics of heroin hydrolysis in dog plasma were studied with and without the enzyme inhibitors sodium fluoride and tetraethyl pyrophosphate. An aliquot (25 ml) of dog plasma was spiked with varying amounts of ^{14}C-labeled heroin in the normal range expected on heroin administration, and 0.5 or 1 ml of this mixture was assayed by liquid scintillation counting for the heroin extracted at pH 4.7 at various times up to 1–2 hr.

The study was repeated with sodium fluoride (20 mg/ml of plasma) and tetraethyl pyrophosphate (34.7 μg/ml of plasma). Similar studies were repeated in 10 ml of fresh (3 hr) dog blood with and without an enzyme inhibitor. Four milliliters of chloroform completely extracted added radiolabeled ^{14}C-heroin from 1.0 ml of plasma. The hydrolysis kinetics of heroin were studied in fresh dog cerebrospinal fluid (6.5 ml) and isotonic phosphate buffer (10 ml) without an enzyme inhibitor.

The stock solutions of heroin and tetraethyl pyrophosphate were prepared in acetone. Appropriate volumes were then transferred to a centrifuge tube, and the solvent was evaporated under a nitrogen stream at room temperature. The dry residue was dissolved in 20 μl of ethanol, and the biological fluids were added.

The hydrolysis kinetics of 6-*O*-acetylmorphine in dog plasma were studied by spiking 10 ml of fresh dog plasma with unlabeled 6-*O*-acetylmorphine (80 μg). Aliquots (1 ml) were taken at 0, 5, 10, 15, 20, and 40 min. The samples were extracted and analyzed by TLC as described previously. Only the 6-*O*-acetylmorphine spot was seen; no spot attributable to morphine was observed.

Plasma Protein Binding of Heroin—The binding of heroin in heparinized dog plasma was determined by ultrafiltration (18). Standard radiolabeled heroin solutions (9–5500 ng/ml) were prepared in enzyme-inhibited (tetraethyl pyrophosphate, 100 or 115 μg/ml) plasma and plasma water. An aliquot (0.5 ml) was taken prior to centrifugation to determine the total radioactivity. The plasma (3 ml) was put in ultrafiltration cones and centrifuged at 3000 rpm for about 10 min until half of the plasma was filtered. The radioactivity of 0.5 ml of the ultrafiltrate was determined. The membrane binding of heroin was calculated similarly by the ultrafiltration of spiked samples of plasma water prepared from the ultrafiltration of plasma.

Red Blood Cell–Plasma Partitions of Heroin—Pseudoplasma was prepared by ultrafiltration of dog plasma through the membrane cones. Aliquots of a previously analyzed ^{14}C-heroin solution were added to pseudoblood, prepared by adding packed red blood cells (1.00 ml) to this pseudoplasma (1.00 ml) containing the tetraethyl pyrophosphate inhibitor. The red blood cells, obtained from the centrifugation of heparinized dog blood, were washed twice with isotonic saline and recentrifuged. No significant hemolysis was observed.

The mixture was then mixed gently in a centrifuge tube and allowed to equilibrate, usually for 20 min. A hematocrit was taken, and the tube was usually centrifuged for 10 min at 1500 rpm. An aliquot (0.2 ml) of the separated plasma was transferred into a vial for radioactivity determination by liquid scintillation counting. The partition coefficients between plasma water and red blood cells were determined also as functions of drug concentration and time.

The study was repeated with a given concentration of ^{14}C-heroin in a synthetic blood prepared from dog plasma and packed washed blood cells.

HPLC Separation of Heroin, 6-*O*-Acetylmorphine, and Morphine—Reversed-phase HPLC was used to separate heroin, 6-*O*-acetylmorphine, morphine, and normorphine by injection of an aliquot of a mixture of the three alkaloids at a flow rate of 2 ml/min. The compounds were monitored by UV detection, with a bonded phase column[12] used for separation. Solvent systems were 60:40 or 55:45 methanol–buffer where the buffer contained 0.1% $(NH_4)_2CO_3$ and 0.01 *M* $(NH_4)_2HPO_4$.

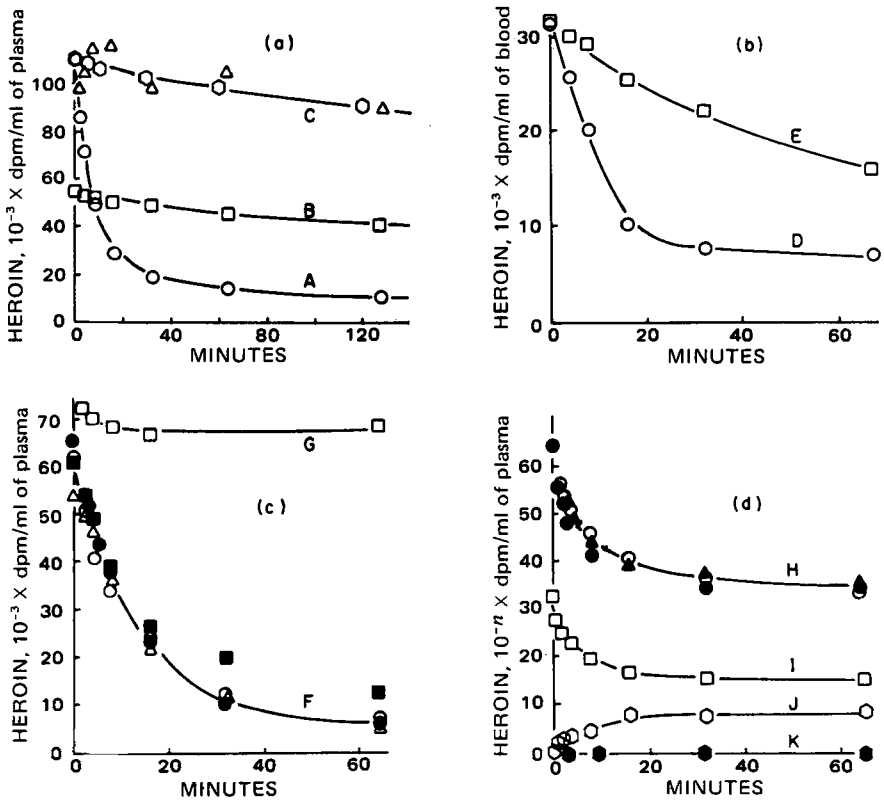

Figure 3—*Instability of heroin in various fluids at room temperature under various conditions. (a) ^{14}C-Heroin (1.2 μg/ml at a specific activity of 91.06 dpm/ng) was completely extracted with chloroform at pH 4.7, and the radiolabeled heroin was assayed by liquid scintillation counting. Key: A (○), fresh dog plasma, no inhibitor; B (□), fresh dog plasma containing 20 mg of sodium fluoride/ml and diluted 1:1 with 0.004 M phosphate buffer (pH 7.4) to give disintegrations per minute per 0.5 ml of plasma; and C, fresh dog plasma (○) containing 34.7 μg of tetraethyl pyrophosphate/ml or 0.004 M phosphate buffer (pH 7.4) containing no inhibitor (△). (b) ^{14}C-Heroin (1.36 μg/ml of blood at a specific activity of 26.9 dpm/ng) was extracted from fresh dog blood with chloroform at pH 4.7, and the radiolabeled heroin was assayed by liquid scintillation counting. Key: D (○), fresh dog blood containing no inhibitor; and E (□), fresh dog blood containing 10 mg of sodium fluoride/ml of blood. (c) Curve F is for ^{14}C-heroin (72 × 10^3 dpm/ml of fresh dog plasma without inhibitors with time as determined by liquid scintillation counting assay of chloroform extraction at pH 4.7). Key: ○, 0.79 μg/ml of plasma; ■, 3.11 μg/ml of plasma; △, 5.43 μg/ml of plasma; and ●, 7.71 μg/ml of plasma. Curve G is for ^{14}C-heroin [82 × 10^3 dpm/ml of fresh dog cerebrospinal fluid without inhibition as determined by liquid scintillation counting assay of benzene–butanol (8.5:1.5) extract]. (d) Curve H, n = 2, is for ^{14}C-heroin (0.216 μg/ml of plasma at a specific activity of 29.9 dpm/ng) extracted from dog plasma of pH 4.7 with 20 ml of benzene–butanol (8.5:1.5)/ml of fresh dog plasma without inhibition. The extract was assayed by liquid scintillation counting. Key: ●, heroin alone; ○, heroin plus 1.6 μg of cold 6-O-acetylmorphine/ml of plasma; and ▲, heroin plus 1.6 μg of cold morphine/ml of plasma. Ethanol (10 μl) had been added to reconstitute evaporated organic solutions of drugs before addition of 25 ml of plasma. ^{14}C-Heroin (1.08 μg/ml of fresh dog plasma at a specific activity of 29.9 dpm/ng) was assayed (n = 3) as for curve H with time (curve I, □). 6-O-Acetylmorphine (curve J, ○) and morphine (curve K, ●) were assayed (n = 3) by liquid scintillation counting of the second extraction after pH adjustment of the heroin-extracted plasma to pH 8.9.*

RESULTS AND DISCUSSION

Separation and Analysis of Heroin and Metabolites—Cold methods for the analyses of morphine after efficient extraction from biological fluids by GLC with electron-capture detection of pentafluoropropionyl derivatives were reported recently (19). The morphine glucuronide metabolite, readily separated from the extracted morphine, was completely hydrolyzed to, and assayed as, morphine.

Heroin and its metabolites (6-*O*-acetylmorphine, morphine, and morphine glucuronide) in biological fluids such as plasma can be analyzed in the same way as morphine after: (*a*) selective sequential extraction procedures as given under *Experimental*, (*b*) TLC separation with subsequent elution of spots at referenced R_f values, and (*c*) separation with HPLC systems. In the HPLC case, UV monitoring can give direct assay of the separated peaks at higher concentrations. Alternatively, the appropriate retention volumes can be collected, acid hydrolyzed to morphine, and assayed by the more sensitive morphine assay methods reported previously (19).

Extraction Efficiencies—Radiolabeled heroin (97% pure by HPLC) was added to plasma where the heroin was inhibited from plasmolysis by added tetraethyl pyrophosphate. Chloroform (4 ml) completely extracted the radioactivity from plasma (1 ml) at an adjusted plasma pH of 4.7.

Benzene–butanol (8.5:1.5), 20 ml, was used to extract ^{14}C-heroin in tetraethyl pyrophosphate-inhibited (173 μg/ml) plasma (1 ml + 1 ml of pH 4.7 buffer), and the organic phase was assayed for radioactivity by liquid scintillation counting. The extraction efficiency was 86.3 ± 1.1% (*SD*) and was independent of the concentration of heroin in the plasma in the range of 285–2280 ng/ml. The specific values were (nanograms of heroin per milliliter of plasma and extraction efficiency): 285, 86.0%; 570, 86.1%; 1140, 84.8%; 1710, 87.6%; and 2280, 87.04%. When the same procedures were conducted with ^{14}C-morphine in plasma, 7 and 9% of the total radioactivity were extracted at pH 4.8.

^{14}C-Morphine was extracted twice from 1 ml of plasma (0.66 μg/ml), mixed with 1 ml of pH 4.8 acetate buffer and 2 ml of pH 8.89 carbonate buffer, by 5 ml of ethyl acetate–isopropyl alcohol (8.5:1.5). The organic phase was then assayed for radioactivity by liquid scintillation counting. The extraction efficiency was 97.53 ± 0.23% (*SD*). In a second study, replicated four times, 0.5 ml of plasma, mixed with 1.5 ml of carbonate buffer (pH 8.9), was extracted with 5 ml of the same organic solvent. The extraction efficiency was 97.31 ± 0.91% (*SE*).

The extraction efficiency of morphine under the conditions for hydrolysis of the morphine glucuronide to morphine was studied also. ^{14}C-Morphine (0.66 μg) was added to 1 ml of plasma, which was mixed with 1 ml of pH 4.7 acetate buffer, 2 ml of pH 8.9 carbonate buffer, and 4 ml of 6 *N* HCl to which 1.15 mg of solid sodium carbonate was added, with the pH adjusted to 8.9. This mixture was extracted with 30 ml of benzene–butanol (8.5:1.5), the organic solution was evaporated, the

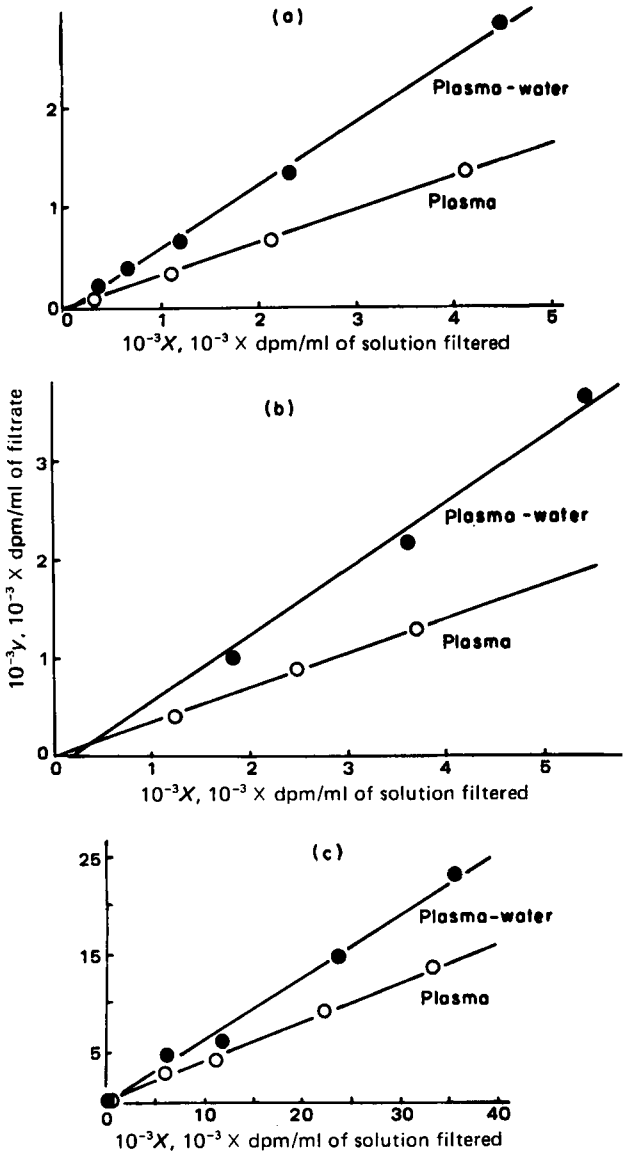

Figure 4—*Plots of disintegrations per minute per milliliter in ultrafiltrate (y) against disintegrations per minute per milliliter of filtered plasma and plasma water solution (x) of ^{14}C-heroin whose hydrolysis was inhibited with tetraethyl pyrophosphate (108 or 115 μg/ml). The drawn lines are those fitted by linear regression in accordance with* $y(\pm SE_y) = m(\pm SE_m)x + b(\pm SE_b)$, *where the* SE_i*'s are the standard errors of estimate. The coefficient of determination is* r^2. *(a) 9–144 ng of ^{14}C-heroin/ml; specific activity = 29.9 dpm/ng. Plasma water:* $y(\pm 60.3) = 0.640(\pm 0.018)x - 57(\pm 42)$; $r^2 = 0.9976$. *Plasma:* $y(\pm 26) = 0.338(\pm 0.009)x - 32(\pm 21)$; $r^2 = 0.9986$. *(b) 180–550 ng of ^{14}C-heroin/ml; specific activity = 6.11 dpm/ng. Plasma water:* $y(\pm 165) = 0.664(\pm 0.041)x - 103(\pm 139)$; $r^2 = 0.9925$. *Plasma:* $y(\pm 10) = 0.345(\pm 0.004) - 0.04(\pm 9)$; $r^2 = 0.9998$. *(c) 920–5500 ng of ^{14}C-heroin/ml; specific activity = 6.11 dpm/ng. Plasma water:* $y(\pm 1056) = 0.633(\pm 0.037)x - 100(\pm 740)$; $r^2 = 0.9900$. *Plasma:* $y(\pm 214) = 0.402(\pm 0.008)x + 42(\pm 150)$; $r^2 = 0.9988$.

residue was reconstituted in 0.5 ml of methanol, and the radioactivity was assayed by liquid scintillation counting. The recoveries of radioactivity were 91.1 and 93.1%.

TLC Separation and Extraction Efficiencies of Developed TLC Spots—Development of mixtures and separate solutions on a TLC silica gel plate with chloroform–methanol (80:20) widely separated morphine (R_f 0.22) from 6-*O*-acetylmorphine (R_f 0.50) and heroin (R_f 0.77). The morphine glucuronide stayed at the origin. ^{14}C-Heroin, 14.5, 36.2, 72.4, 108.6, and 144.7 ng, was spotted and developed on TLC plates. The developed spots were scraped, the scrapings were extracted with 0.5 ml of methanol, and the radioactivity was assayed by liquid scintillation counting. The extraction efficiency from the scrapings was 80.9 ± 1.2% (*SD*) and was independent of the amount spotted (Fig. 1).

Similar studies with spotted ^{14}C-morphine gave an extraction efficiency of 82.3 ± 1.1% (*SD*).

HPLC Separations—Several HPLC systems were developed for separation and analyses of morphine and its metabolites. A typical separation for normorphine, morphine, 6-*O*-acetylmorphine, and heroin by 55:45 methanol–buffer, with the pH 6.98 buffer containing 0.1% $(NH_4)_2CO_3$ and 0.01 *M* $(NH_4)_2HPO_4$, is given in Fig. 2. When the purified synthesized ^{14}C-heroin was analyzed by HPLC in such a system, 96.5% of the administered radioactivity was collected at the retention volume of heroin (9.5–13 min), 2.49% at that of morphine (2.5–4 min), and 0.83% at that of 6-*O*-acetylmorphine (4.5–6.5 min).

Organic solvent extracts of inhibited plasma (1 ml), obtained with 20 ml of ethyl acetate–isopropyl alcohol or benzene–butanol (8.5:1.5) from the plasma mixed with 1 ml of pH 8.9 carbonate buffer, contained heroin, 6-*O*-acetylmorphine, and morphine. The extracts were reconstituted in 50 μl of acetone, of which 10 μl was injected in the liquid chromatograph and developed by the previously stated procedures. The fractions were monitored directly by UV, using the peak heights, peak areas, or their ratios to the respective values of an internal standard such as normorphine.

In the apparatus used, the sensitivity of the direct UV assay was 0.1 μg/injection (inset, Fig. 2) and thus equivalent to 0.5 μg/ml of plasma. Greater sensitivity could be obtained by acid hydrolysis of the collected pertinent fractions with assay as morphine by the previously described (19) GLC method for the derivatized compound or liquid scintillation counting of the radiolabeled dansyl derivatization product. Direct liquid scintillation counting could assay the fraction if ^{14}C-heroin were administered to the animal.

Instability of Heroin and Its Inhibition in Plasma—Heroin underwent some spontaneous hydrolysis in pH 7.4 phosphate buffer (curve C, Fig. 3*a*) with an estimated apparent half-life of 415 min. The hydrolysis accelerated in fresh dog plasma with an apparent half-life of 8 min (curve A, Fig. 3*a*). The presence of 20 mg of sodium fluoride/ml of plasma (curve B, Fig. 3*a*) and 34.7 μg of tetraethyl pyrophosphate/ml of plasma (curve C, Fig. 3*a*) reduced the hydrolysis rate to that of buffer. The instability of heroin in fresh cerebrospinal fluid (curve G, Fig. 3*c*) was close to that in buffer alone (curve C, Fig. 3*a*).

The addition of 10 mg of sodium fluoride/ml to dog blood significantly increased the estimated half-life of heroin instability from 8 min (curve D, Fig. 3*b*) to 40 min (curve E, Fig. 3*b*). Unfortunately, sodium fluoride ruptured red blood cells and denatured plasma protein. Thus, tetraethyl pyrophosphate was the inhibitor of choice.

Additional studies of heroin instability in other dog plasma (curve F, Fig. 3*c*) also showed apparent half-lives of 8 min and were independent of heroin concentrations in the studied range of 0.79–7.7 μg/ml of plasma.

When these plasmas (curve F, Fig. 3*c*) were kept at room temperature for 24 hr and respiked with heroin, no significant loss of the added heroin was observed. The 24-hr sample that originally had 0.79 μg of heroin/ml of plasma was adjusted to 416,700 dpm/ml assayed as heroin and, 10 min later, the assayed heroin was still 478,500. Similarly, the original 7.71 μg/ml of plasma was adjusted to 478,500 dpm/ml with ^{14}C-heroin, and the count of assayed extract after 10 min was 481,000 dpm/ml. Also, negligible hydrolysis of ^{14}C-heroin was observed in stored plasma in several instances. These studies imply that the catalysts or enzymes in plasma that promote heroin hydrolysis are inactivated on storage.

When similar studies were conducted with fresh dog plasma that was not stored for such a long interval, the catalytic activity was maintained. A plasma containing 1037 ng/ml assayed 83,760 dpm/ml as heroin at zero time and 8112 at 63 min. When additional ^{14}C-heroin, 1570 μg/ml, was added to the plasma, the assays were 128,500 dpm/ml at 65 min and 23,216 dpm/ml at 125 min.

For several plasmas stored for varying times, incompletely catalyzed hydrolysis was observed (curve H, Fig. 3*c*). The possiblity of product-inhibited hydrolysis was denied by the demonstration that heroin alone, heroin plus 6-*O*-acetylmorphine, and heroin plus morphine demonstrated the same kinetics of heroin hydrolysis in plasma (curve H, Fig. 3*c*).

The major observed product of plasma-catalyzed heroin hydrolysis was 6-*O*-acetylmorphine; negligible radioactivity assignable to morphine was observed (curves I–K, Fig. 3*d*). A specific study was made of the attempted hydrolysis of 6-*O*-acetylmorphine (8 μg/ml) in fresh plasma and plasma inhibited with tetraethyl pyrophosphate. Extraction with 5 ml of ethyl acetate–isopropyl alcohol (8.5:1.5) of 1.00-ml plasma samples adjusted to pH 8.9 with 2 ml of carbonate buffer with subsequent solvent evaporation, reconstitution in 25 μl of ethyl acetate–isopropyl alcohol,

Table I—Summary of Membrane and Plasma Protein Binding Studies of ^{14}C-Heroin by Ultrafiltration

Heroin Concentration, ng/ml	Tetraethyl Pyrophosphate Concentration, μg/ml	Assay Levels	Assay Level Replicates	Specific Activity, dpm/ng	Fraction in Plasma Water Not Bound to Membrane ±*SD*[a] (a)	Fraction in Plasma: Not Bound to Membrane and Plasma Protein[b] ±*SD* (b)	Fraction in Plasma: Not Bound to Plasma Protein[c] (c)	Fraction of Free Heroin Bound to Plasma Protein[d]
9–144	108	5	3	29.9	0.596 ± 0.032	0.313 ± 0.016	0.283	0.475
21–127	108	3	1	39.0	0.690 ± 0.008	0.470 ± 0.018	0.220	0.319
293	115	1	4	6.81	0.655	0.372	0.283	0.432
183–550	115	3	3	6.11	0.609 ± 0.054	0.344 ± 0.05	0.265	0.435
920–5500	115	4	3	6.10	0.64* ± 0.12	0.411 ± 0.029	0.231	0.360
Average ± *SD*					0.64 ± 0.04	0.38 ± 0.06	0.26 ± 0.03	0.40 ± 0.06

[a] Disintegrations per minute per milliliter of plasma water filtrate ÷ disintegrations per minute per milliliter of plasma water before filtration. Assayed values in plasma water before and after filtration were used in all cases. [b] Disintegrations per minute per milliliter of plasma filtrate ÷ disintegrations per minute per milliliter of plasma before filtration. Assayed values in plasma were used in all cases except for the 920–5500-ng/ml plasma studies where the disintegrations per minute per milliliter of plasma before filtration were calculated from the known specific activity and the amount of ^{14}C-heroin added to the plasma. [c] Calculated from a − b. [d] Calculated from (a − b)/a = (c/a).

and TLC development of 15 μl showed only the presence of 6-*O*-acetylmorphine and no morphine after 0, 5, 10, 15, 20, and 40 min of reaction in the plasma. This result strongly indicates that only heroin hydrolysis is catalyzed by fresh plasma.

Plasma Protein Binding of ^{14}C-Heroin by Ultrafiltration—The ultrafiltration of ^{14}C-heroin from tetraethyl pyrophosphate-inhibited plasma water and plasma demonstrated linear relations between the concentrations of radiolabeled material in the filtrate and the solutions filtered (Fig. 4), with intercepts not significantly different from zero. The respective slopes were estimates of the fraction unbound to membrane, S_{PW}, and unbound to plasma proteins plus membrane, S_P, for the filtration of plasma water and plasma, respectively. These plots showed that the fractions membrane bound, $1 - S_{PW}$, were 0.360 ± 0.018 (*SE*) for 9–144 ng of heroin/ml, 0.336 ± 0.041 for 180–550 ng of heroin/ml, and 0.367 ± 0.037 for 920–5500 ng of heroin/ml. The fractions, both membrane and plasma protein bound, $1 - S_P$, were 0.662 ± 0.009, 0.655 ± 0.004, and 0.598 ± 0.037, respectively. Thus, it can be concluded that the fractions bound of heroin were concentration independent in the 9–550-ng/ml range. The fractions of heroin not bound to the membrane but bound to plasma protein, $(S_{PW} - S_P)/S_{PW}$, were 0.472, 0.480, and 0.365, respectively.

A summary of membrane and plasma protein binding for all studies conducted, calculated from the averages of the data, is given in Table I. The values were calculated differently from the methods given in Fig. 4. The overall average of the fraction of free heroin, *i.e.*, unbound to membrane, that was bound to plasma protein was 0.40 ± 0.06 *SD*.

Additional studies were conducted at 1467 ng of ^{14}C-heroin/ml of plasma; aliquots of the plasma were filtered at 0, 4, 8, 16, and 32 min. There were no significant differences among the assays of the filtrates obtained at different times. In two studies, the fractions bound to membrane plus plasma proteins were 0.624 ± 0.049 *SD* and 0.647 ± 0.017 *SD*, $n = 5$. It must be concluded that equilibration between plasma protein and heroin was established well within the time necessary for processing the plasma.

Table II—Summary of Red Blood Cell–Plasma Water Partition Studies[a]

Partition System	*H*, hematocrit/100	ng of ^{14}C-Heroin / ml of System	A_B, dpm of ^{14}C-Heroin / ml of System	A_P, dpm of ^{14}C-Heroin / ml of Plasma or Plasma Water	*D*[b] (Partition Coefficient), RBC/plasma water	Equilibration Time, min	Centrifugation Time, min
A Plasma water–red blood cells	0.345	38.0	1532	1320	1.47	20	10
	0.356	59.6	2298	2010	1.41	20	10
	0.350	75.4	3064	2560	1.56	20	10
	0.350	94.9	3830	3155	1.61	20	10
	0.352	94.9	3830	3210	1.55	20	10
	0.350	94.9	3830	3140	1.63	20	10
	0.345	190	7660	6755	1.39	20	10
Average ± *SD*					1.52 ± 0.10		
B Plasma water–red blood cells	0.345	94.9	3830	3320	1.45	2	10
	0.345	94.9	3830	3390	1.38	0	10
Average					1.41		
C Plasma water–red blood cells	0.301	94.9	3830	3130	1.74	0	3
	0.295	94.9	3830	3510	1.31	4	3
	0.296	94.9	3830	3465	1.36	8	3
	0.298	94.9	3830	3425	1.40	16	3
	0.295	94.9	3830	3837	0.99	32	3
Average ± *SD*					1.36 ± 0.27		
D Plasma water–red blood cells	0.300	39.3	1523	1381	1.34	20	10
	0.300	39.3	1523	1499	1.05	20	10
	0.300	39.3	1523	1480	1.10	20	10
	0.305	39.3	1523	1503	1.04	20	10
Average ± *SD*					1.13 ± 0.14		
E Plasma water–red blood cells	0.232	16.8	689	568	1.92	20	10
	0.228	16.8	648	598	1.37	20	10
	0.233	16.8	655	599	1.40	20	10
	0.235	16.8	647	568	1.59	20	10
Average ± *SD*					1.57 ± 0.25		
F Plasma–red blood cells	0.253	104.5	1892	1947	1.48	20	10
	0.285	104.5	1892	2030	1.27	20	10
	0.268	104.5	1892	1963	1.44	20	10
	0.274	104.5	1892	2037	1.23	20	10
Average ± *SD*					1.36 ± 0.12[c]		

[a] Plasma water–red blood cell systems were inhibited with 86.5 μg of tetraethyl pyrophosphate/ml. The plasma–red blood cell system was inhibited with 104.5 μg of tetraethyl pyrophosphate/ml. The fraction bound, *f*, was taken as zero for the plasma water–red blood cells systems and as 0.4 for the plasma–red blood cells system. [b] Average of averages was 1.39 ± 0.15 *SD*. [c] If $f = 0$ were taken rather than $f = 0.4$, the calculated *D* would be 0.814 ± 0.074 *SD*.

Partitioning of ^{14}C-Heroin between Plasma Water and Red Blood Cells—The results of the several studies conducted on the partitioning of ^{14}C-heroin between plasma water and red blood cells (RBC) are summarized in Table II. The heroin in the systems was inhibited from plasmolysis with 86.5 or 104.5 μg of tetraethyl pyrophosphate/ml. The partition coefficient, D, was calculated from (20):

$$D = \frac{[A_{RBC}]}{[A_p^u]} = \left\{ \frac{[A_B]}{[A_p](1-H)(1-f)} - \frac{f}{1-f} - 1 \right\} \frac{1-H}{H} \quad \text{(Eq. 1)}$$

where $[A_{RBC}]$, $[A_B]$, and $[A_p]$ are the ^{14}C-heroin concentrations in the red blood cells, blood, and plasma, respectively, and $[A_p^u]$ is the drug concentration in the plasma that is unbound to plasma proteins. The hematocrit ÷ 100 is H, and f is the fraction of drug in plasma bound to plasma proteins. The value of f was taken as zero in the plasma water systems and as 0.4 in the plasma–red blood cell mixture.

The results of Study A (Table II) showed no significant differences among the partition coefficients, D, within the range of 38–190 ng of ^{14}C-heroin/ml of pseudoblood. Any time of equilibration greater than zero for ^{14}C-heroin in the pseudoblood did not affect significantly the determined partition coefficients (compare Studies A–C in Table II). Also, times of centrifugation, 3 and 10 min, of the plasma water–red blood cell mixtures to separate the plasma water to be assayed did not significantly affect the results. Only Study D appeared to differ slightly from the total results where the average of the averages given in Table II was 1.39 ± 0.15 *SD*.

Study E was performed after separation of the plasma water from Study D, where the red blood cells containing the equilibrated ^{14}C-heroin were reequilibrated with fresh plasma water and subsequently centrifuged to determine the new partition coefficient. Study E was on a synthetic blood, prepared from plasma and red blood cells, and the partition coefficient determined from Eq. 1 for a protein-bound fraction of $f = 0.4$ was the same as that from the plasma water–red blood cell systems. If the fraction bound had been ignored, *i.e.*, $f = 0$, the calculated apparent D would have been 0.814 ± 0.074 *SD*, which differs widely from the plasma water systems since the protein binding makes a fraction of the heroin unavailable for partition into the red blood cells.

REFERENCES

(1) C. I. Wright, *J. Pharmacol. Exp. Ther.*, **71**, 164 (1947).

(2) E. L. Way, J. W. Kemp, J. M. Young, and D. R. Grassetti, *ibid.*, **129**, 144 (1960).

(3) E. L. Way, J. M. Young, and J. W. Kemp, *Bull. Narc.*, **17** (1), 15 (1965).

(4) T. M. Shen, *J. Clin. Pharm. Assoc.*, **1**, 124 (1936); through *Chem. Abstr.*, **31**, 730 (1937).

(5) F. W. Oberst, *J. Pharmacol. Exp. Ther.*, **73**, 401 (1941).

(6) F. W. Oberst, *ibid.*, **79**, 266 (1943).

(7) P. N. Mo and E. L. Way, *ibid.*, **154**, 142 (1966).

(8) H. W. Elliott, K. D. Parker, J. A. Wright, and N. Nomoff, *Clin. Pharmacol. Ther.*, **12**, 806 (1971).

(9) S. Y. Yeh and R. L. McQuinn, *J. Pharm. Sci.*, **64**, 1237 (1975).

(10) S. Y. Yeh, C. W. Gorodetzky, and R. L. McQuinn, *J. Pharmacol. Exp. Ther.*, **196**, 249 (1976).

(11) S. Y. Yeh, R. L. McQuinn, and C. W. Gorodetzky, *J. Pharm. Sci.*, **66**, 201 (1977).

(12) G. R. Nakamura, J. I. Thornton, and T. T. Noguchi, *J. Chromatogr.*, **110**, 81 (1975).

(13) S. Ellis, *J. Pharmacol. Exp. Ther.*, **94**, 130 (1948).

(14) G. L. Cohn, J. A. Cramer, and H. D. Kleber, *Proc. Soc. Exp. Biol. Med.*, **144**, 351 (1973).

(15) G. L. Cohn, J. A. Cramer, W. McBride, R. C. Brown, and H. D. Kleber, *ibid.*, **147**, 664 (1974).

(16) E. Stahl, "Thin Layer Chromatography," Springer-Verlag, New York, N.Y., 1965, p. 493.

(17) S. E. Hays, L. T. Grady, and A. V. Kruegel, *J. Pharm. Sci.*, **62**, 1509 (1973).

(18) P. H. Hinderling, J. Brès, and E. R. Garrett, *ibid.*, **63**, 1684 (1974).

(19) E. R. Garrett and T. Gürkan, *ibid.*, **67**, 1512 (1978).

(20) E. R. Garrett and H. J. Lambert, *ibid.*, **62**, 550 (1973).

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Liquid Chromatography in Pharmaceutical Analysis XI: Determination of Muscle Relaxant–Analgesic Mixtures Using Reversed-Phase and Ion-Pair Techniques

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Abstract □ High-pressure liquid chromatography using reversed-phase and/or ion-pair techniques was used to optimize resolution of aspirin-containing muscle relaxant mixtures as well as other therapeutic agents commonly found in muscle relaxant–analgesic mixtures. The compounds were chromatographed on an octadecylsilane column using methanol–water solvent systems, some of which contained tetrabutylammonium cation as counterion. Mixtures of methocarbamol–aspirin and chlorzoxazone–acetaminophen were selected to demonstrate separation and quantification. The methocarbamol–aspirin mixture was chromatographed with methanol–water (40:60, pH 6.8) containing 0.01 *M* tetrabutylammonium cation at a flow rate of 2.0 ml/min. The chlorzoxazone–acetaminophen mixture was chromatographed with methanol–water (50:50) at a 2.0-ml/min flow rate. The separation and quantitation of each mixture were achieved in approximately 8 min with accuracy in the 2–3% range.

Keyphrases □ Muscle relaxant–analgesic mixtures, various—high-pressure liquid chromatographic analyses of components □ Analgesic–muscle relaxant mixtures, various—high-pressure liquid chromatographic analyses of components □ High-pressure liquid chromatography—analyses, components of various muscle relaxant–analgesic mixtures

Previous high-pressure liquid chromatographic (HPLC) investigations with muscle relaxant–analgesic agents such as meprobamate, methocarbamol, carisoprodol, chlorzoxazone, acetaminophen, aspirin, caffeine, and phenacetin (1) indicated that the aspirin-containing combinations were difficult to chromatograph using nonpolar solvents

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