(6) A. W. Hixon and J. H. Crowell, *Ind. Eng. Chem.*, **23**, 923 (1931).
(7) A. W. Hixon and J. H. Crowell, *Ind. Eng. Chem.*, **23**, 1002 (1931).
(8) J. Cobby, M. Mayersohn, and G. C. Walker, *J. Pharm. Sci.*, **63**, 725 (1974).
(9) J. Cobby, M. Mayersohn, and G. C. Walker, *J. Pharm. Sci.*, **63**, 732 (1974).
(10) S. Mishima and S. Nagataki, *Contact Intraocular Lens Med. J.*, **4**(3), (Conrad Berens Memorial Lecture; Pharmacology of Ophthalmic Solutions), 23 (1978).
(11) S. S. Chrai, T. F. Patton, A. Mehte, and J. R. Robinson, *J. Pharm. Sci.*, **62**, 1112 (1973).

Electrochemical Chromatographic Determinations of Morphine Antagonists in Biological Fluids, with Applications

HARTMUT DERENDORF, ALAA EL-DIN A. EL-KOUSSI, and EDWARD R. GARRETT ˣ

Received December 17, 1982, from *The Beehive, College of Pharmacy, J. Hillis Miller Health Center, University of Florida, Gainesville, FL 32610.* Accepted for publication January 27, 1983.

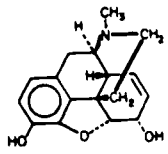
Abstract □ The morphine antagonists naltrexone and naloxone were extracted from plasma and urine, separated on a chromatographic column, and assayed by electrochemical detection. Optimum oxidation potentials were 0.65 V for morphine and 0.75 V for naloxone and naltrexone. Assay sensitivities were 2–5 ng/mL for plasma and 10 ng/mL for urine. The assays were applied to determine red blood cell partition coefficients of 1.83 ± 0.15 (*SD*) for naltrexone and 1.49 ± 0.27 (*SD*) for naloxone in a concentration range of 10–3500 ng/mL. No significant time dependence for the partitioning could be observed. Plasma protein binding in the same concentration range, determined by ultracentrifugation, was 27.7% ± 2.5% (*SD*) for naltrexone and 30.1% ± 5.1% (*SD*) for naloxone. The degree of protein binding did not change in the presence of morphine for morphine–antagonist ratios between 1:10 and 10:1. No concentration dependencies of red blood cell partitioning or protein binding were observed.

Keyphrases □ Naltrexone—HPLC with electrochemical detection, plasma and urine, applications to protein binding and red blood cell partitioning □ Naloxone—HPLC with electrochemical detection, plasma and urine, applications to protein binding and red blood cell partitioning □ Protein binding—naltrexone and naloxone in the presence of morphine, HPLC with electrochemical detection, red blood cell partitioning □ Red blood cell partitioning—naltrexone and naloxone in the presence of morphine, HPLC with electrochemical detection, protein binding

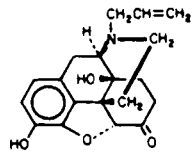
The classical detection methods for high-performance liquid chromatography (HPLC) by spectrophotometric and fluorescence detection are not useful for some drugs with low absorptivities or low fluorescence. Electrochemical detection has been a method of recent choice in selected instances. It combines amperometric titration and liquid chromatography (1) and has been applied to the assays of catecholamines (2), phenolic compounds (3), phenothiazines (4, 5), and morphine (I) (6–8).

The present study describes sensitive and accurate HPLC assays for the antagonists of morphine, naloxone (II) and naltrexone (III) in biological fluids with electrochemical detection. Studies on possible pharmacokinetic interactions between morphine and its antagonists are needed since naltrexone or naloxone are given to morphine-overdosed subjects. Prerequisites for such studies are specific and sensitive assays that allow simultaneous determinations of agonist and antagonist as well as their metabolites.

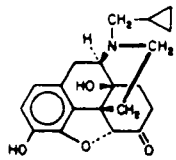
A facile procedure for the simultaneous determination of I and III or II is described using HPLC with electrochemical detection. The assay was applied to the determination of the protein binding of II and III and the influence of I on their protein binding. The red blood cell partition properties of both antagonists were also investigated.



I



II



III

EXPERIMENTAL

Materials—The following analytical-grade materials were used: sodium bicarbonate[1], monobasic potassium phosphate[1], volumetric concentrates of hydrochloric acid and sodium hydroxide[2], 1-butanol[3], and benzene[4]. Acetonitrile[4] was HPLC grade. Morphine (I)[5], naloxone (II)[5], and naltrexone (III)[5] were used as received. Sodium chloride injection USP[6], sodium heparin injection[7], and disposable syringes[8] were used in the preparation of red blood cell suspensions.

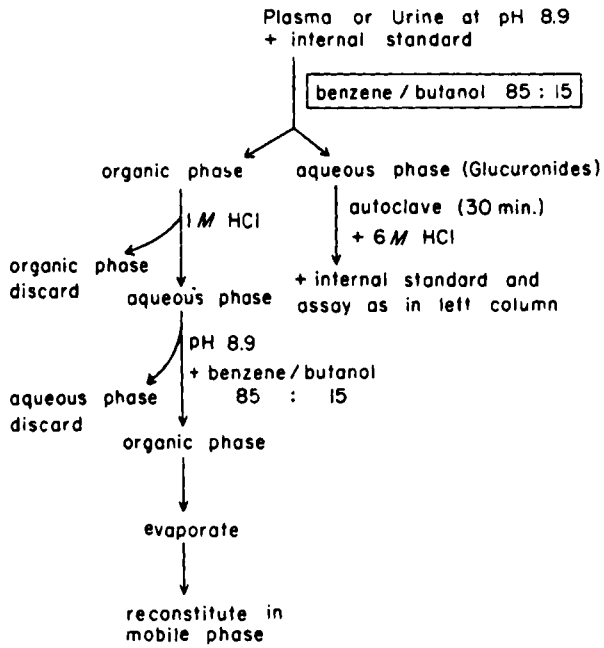
Apparatus—For the HPLC assay the following instruments were used: high-pressure pump[9], a six-port injector[10], an electrochemical detector[11] with a working electrode of glassy carbon[12], a strip-chart recorder[13], and an octadecylsilane column[14] with a guard column of the same material.

Plasma protein binding was determined with an ultracentrifuge[15]. A laboratory centrifuge[16] was used in the separation of organic extracts from aqueous phases.

Chromatographic Conditions—The mobile phase was a mixture of 0.04 M monobasic potassium phosphate and acetonitrile (90:10). The flow rate was

[1] Mallinckrodt, Paris, Ky.
[2] Ricca Chemical Co., Arlington, Tex.
[3] Burdick & Jackson Laboratories, Muskegon, Mich.
[4] Fisher Scientific Co., Fair Lawn, N.J.
[5] National Institute on Drug Abuse, Research Technology Branch, Rockville, Md.
[6] McGaw Laboratories, Irvine, Calif.
[7] The Upjohn Co., Kalamazoo, Mich.
[8] Monoject, Division of Sherwood Medical, A. Brunswick Co., St. Louis, Mo.
[9] Series 3B Microcomputer controlled pump module; Perkin-Elmer, Norwalk, Conn.
[10] Model 7105; Rheodyne, Cotati, Calif.
[11] LC 4A; Bioanalytical Systems, West Lafayette, Ind.
[12] TLS; Bioanalytical Systems, West Lafayette, Ind.
[13] Series 5000, Fisher Recordall; Fisher Scientific Co., Fair Lawn, N.J.
[14] C_{18} μBondapak column; Waters Associates, Milford, Mass.
[15] Ultracentrifuge Model LS-50 with rotor Ti 50; Beckman Instruments, Norcross, Ga.
[16] Lab centrifuge; International Centrifuge Equipment Co., Needham, Mass.

0022-3549/84/0500-0621$01.00/0



Scheme I—Extraction procedure for plasma and urine.

1.2 mL/min, with a back pressure of 6 MPa. Electrochemical detection was performed with an applied potential of 0.75 V.

Extraction Procedure from Plasma and Urine—Plasma or urine (1.0 mL) was placed in a tube containing 0.25 mL of 1 M sodium bicarbonate adjusted to pH 8.9 with 1 M NaOH. An appropriate amount of internal standard (II, I, or III depending on the compound assayed) was added to give peak height ratios between 0.2 and 4. A 2-mL volume of benzene–1-butanol (85:15) was added, and the mixture was vortexed for 30 s. The phases were separated by centrifugation for 10 min at 3000 rpm, and the organic phase was transferred with a silylated Pasteur pipet into another tube. The extraction was repeated with 2 mL of fresh solvent.

The aqueous phase can be saved for the analysis of the glucuronides. The combined organic phases were extracted with 0.5 mL of 1 M HCl and discarded. The acidic aqueous phase was washed with 2 mL of fresh organic solvent and then neutralized with 0.5 mL of 1 M NaOH. The pH was adjusted to 8.9 with 1 M sodium bicarbonate buffer, and the solution was twice reextracted with 2 mL of benzene–1-butanol (85:15). The organic phases were combined in a vial and evaporated under a nitrogen stream at 55°C. The dried residue was dissolved in 75 μL of mobile phase, and 10–25 μL was injected into the HPLC system. The extraction procedure is given in Scheme I.

The aqueous phase saved after the first extraction can be assayed for glucuronides of the compounds by the method given for morphine after hydrolysis of the conjugate (9).

Determination of Plasma Protein Binding by Ultracentrifugation—Fresh heparinized dog blood was centrifuged for 15 min at 3000 rpm. Plasma aliquots (5 mL) were spiked with different amounts of III or II to give concentrations between 10 and 3500 ng/mL of plasma. Plasma (1 mL) was taken for analysis before ultracentrifugation. After ultracentrifugation at 35,000

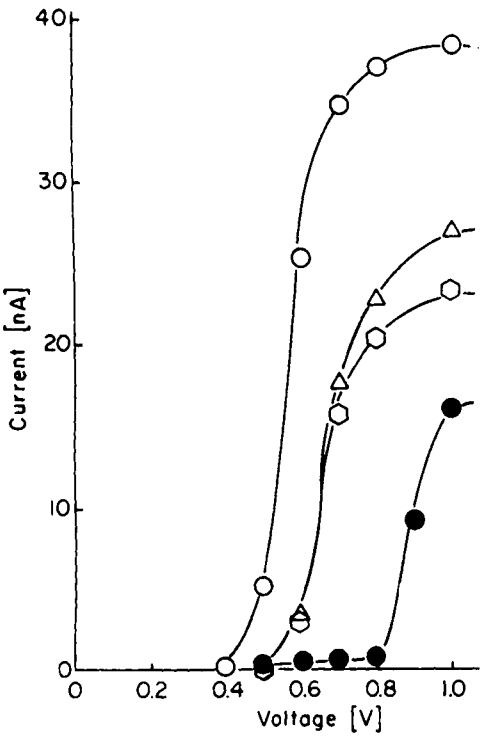


Figure 1—*Chromatographic amperogram for I (○), II (△), III (○), and the background (●) determined by application of different oxidation potentials. The background arises from oxidation of electroactive impurities in the mobile phase.*

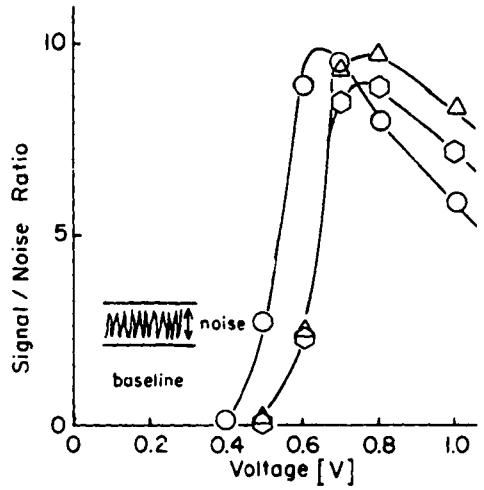


Figure 2—*Determination of optimum oxidation potential by maximization of the signal/noise ratio. Noise was measured as the amplitude of the baseline. The optimum potentials to be applied for detection are 0.65 V for I (○) and 0.75 V for III (○) and II (△).*

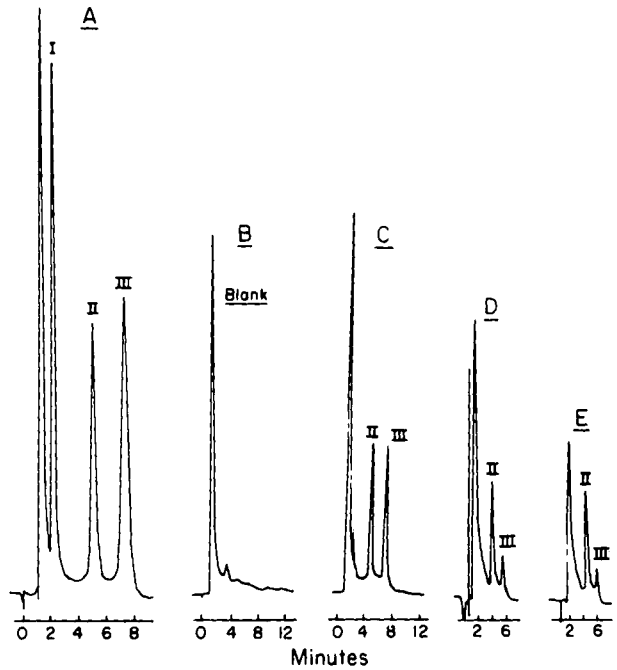


Figure 3—*Chromatograms of I, II, and III. Key: (A) 500 ng/mL in water, injection volume 20 μL; (B) 1 mL of plasma extracted, evaporated to dryness under nitrogen, and reconstituted in 75 μL of mobile phase. Injection volume 20 μL; (C) 1 mL of plasma containing 100 ng/mL of II and III, extracted and treated as in B; (D) 1 mL of plasma containing 10 ng/mL of III extracted with 50 ng/mL of II as internal standard; (E) plasma sample of D after ultracentrifugation for the determination of protein binding.*

Table I—Statistics of Calibration Curves [a]

Compound	Extracted Medium	Internal Standard	C_{IS}, ng/mL	$m \pm s_m$	$b \pm s_b$	$s_{x,y}$ [b]
Naloxone (II)	Plasma	III	30.0	10.73 ± 0.81	2.11 ± 2.34	2.35
Naltrexone (III)	Aqueous Solution	I	100.0	147.95 ± 1.66	2.35 ± 1.36	1.61
	Plasma	II	30.0	71.85 ± 5.24	1.84 ± 2.29	2.29
	Urine	II	100.0	256.47 ± 4.24	3.57 ± 4.96	6.44

[a] Concentrations (C) in ng/mL *versus* peak height ratios (PHR) relative to the internal standard, $C \pm s_{x,y} = (m \pm s_m)\text{PHR} + (b \pm s_b)$ (15). [b] Standard error of estimate y on x, concentration in ng/mL, on peak height ratio.

Table II—Protein Binding of II by Ultracentrifugation

C_{pre}, ng/mL [a]	C_{post}, ng/mL [b]	R [c]	V_{dil}, mL [d]	m [e]	$f_b \cdot 100$, % [f]
10.0	7.5	0.750	0.050	1.010	25.2
35.0	22.1	0.631	0.175	1.035	37.7
100.0	66.4	0.664	0.050	1.010	33.8
350.0	259.0	0.740	0.175	1.035	26.7
1000.0	743.6	0.744	0.050	1.010	25.8
3500.0	2418.5	0.691	0.175	1.035	31.6
Mean ± *SD*					30.1 ± 5.1

[a] Before ultracentrifugation. [b] In plasma water after ultracentrifugation. [c] Ratio between the concentrations after and before ultracentrifugation. [d] Volume of spiked solution added to 5 mL of plasma. [e] Dilution factor $(5 + V_{dil})/5$. [f] Percentage bound to plasma proteins.

Table III—Protein Binding of III by Ultracentrifugation

C_{pre}, ng/mL [a]	C_{post}, ng/mL [b]	$f_b \cdot 100$, % [c]
10.0	7.6	24.2
35.0	25.3	28.4
100.0	73.5	26.7
350.0	253.3	28.3
1000.0	684.2	31.8
3500.0	2592.6	26.6
Mean ± *SD*		27.7 ± 2.5

[a] Before ultracentrifugation. [b] In plasma water after ultracentrifugation. V_{dil} was 0.050–0.175 mL; the dilution factor was $(5 + V_{dil})/5$. [c] Percentage bound to plasma proteins.

rpm for 18 h, 1 mL of the supernatant plasma water was analyzed for III or II. The influence of I on the protein binding of both antagonists was studied in ratios 1:10, 1:2, 2:1, and 10:1.

Red Blood Cell Partition Studies—Fresh heparinized dog blood was centrifuged for 15 min at 2000 rpm. The plasma was removed and isoosmotic phosphate buffer (pH 7.4) was added to the erythrocytes. The red blood cells were gently suspended and centrifuged for 10 min at 2000 rpm; this washing procedure was repeated three times. Red blood cell suspensions in plasma water were spiked with III or II to yield total drug concentrations of 10–3500 ng/mL. The hematocrit was determined routinely using a microcentrifuge with capillary tubes. The spiked suspensions were allowed to equilibrate for 60 min and then centrifuged for 10 min at 2000 rpm. An aliquot of the supernatant solution was analyzed, and the red blood cell partition coefficient was calculated.

For the investigation of a time dependency of the red blood cell partitioning, the procedure was repeated and samples were taken after 4, 6, 8, 10, and 15 min after spiking. A minimum of 4 min of centrifugation is necessary to get a clear supernatant.

To reconfirm the observed red blood cell partition coefficients, the ratio between the drug concentrations in whole blood and plasma were determined. Whole blood (5 mL) was spiked with different amounts of drug to give whole blood concentrations between 10 and 3500 ng/mL. The blood was allowed to equilibrate for 60 min and then centrifuged for 10 min at 3000 rpm. An aliquot of the supernatant plasma was analyzed.

RESULTS AND DISCUSSION

Determination of the Optimum Detection Conditions—Signals for the three compounds were measured after application of voltages between 0.4 and 1.0 V to determine the optimum conditions for the electrochemical detection (Fig. 1). Background is defined as the Faradaic current arising from the oxidation of electroactive impurities in the mobile phase, analogous to the background absorbance in UV detectors. The detection conditions were optimized to find the maximal signal/noise ratio for each of the investigated compounds. Usually measured from peak to peak, the noise is due to pump pulsation, flow hydrodynamics, cell surface reactions, power line noise, and electronic amplification (10). The noise with an electrochemical detector is dependent on the magnitude of the background signal; the greater the background, the greater the noise. The signal/noise ratios for the three compounds are shown in Fig. 2. The maxima of these plots are the optimum potentials to be applied for detection: 0.65 V for I and 0.75 V for II and III.

Chromatographic Separation—With a mobile phase of 0.04 M monobasic potassium phosphate–acetonitrile (90:10) I–III are well separated and can be assayed simultaneously (Fig. 3A). The chromatographic background after extracting blank plasma is clean, so that low concentrations of the compounds can be detected (Fig. 3B, C). Statistics for calibration curves of II and III after extraction from plasma and urine are given in Table I. The limit of assay sensitivity for all compounds was 2–5 ng/mL for plasma and 10 ng/mL for urine. A chromatogram of a plasma sample containing 10 ng/mL of III is shown in Fig. 3D.

Protein Binding of II and III by Ultracentrifugation—The protein binding was determined by the ultracentrifugation method (11) and can be calculated from:

$$f_b = \frac{m(1 - R)}{R + m(1 - R)} \qquad \text{(Eq. 1)}$$

where f_b is the fraction bound to the plasma proteins, R is the ratio of the drug concentration (C_{post}) in plasma water after ultracentrifugation to the concentration (C_{pre}) in plasma before ultracentrifugation, and m is a dilution factor to compensate for the slight dilution of plasma on spiking. A chromatogram of a plasma sample containing 7.6 ng/mL of plasma water after ultracentrifugation is shown in Fig. 3E. The results are summarized in Tables

Table IV—Protein Binding of II and III in the Presence of I at Different Morphine–Morphine Antagonist Ratios

Antagonist	R [a]	C_{pre}, ng/mL [b]	C_{post}, ng/mL [c]	R [d]	V_{dil}, ml [e]	m [f]	$f_b \cdot 100$, % [g]
Naloxone (II)	0.1	98.0	66.1	0.674	0.100	1.020	33.0
	0.5	98.5	72.0	0.731	0.075	1.015	27.2
	2.0	97.1	72.7	0.749	0.150	1.030	25.6
	10.0	98.0	70.3	0.717	0.100	1.020	28.7
Naltrexone (III)	0.1	98.0	71.3	0.727	0.100	1.020	27.7
	0.5	98.5	69.1	0.702	0.075	1.015	30.1
	2.0	97.1	72.0	0.741	0.150	1.030	26.5
	10.0	98.0	70.8	0.722	0.100	1.020	28.2

[a] Ratio of I-antagonist for 100 ng of antagonist/mL of plasma. [b] Before ultracentrifugation. [c] In plasma water after ultracentrifugation. [d] Ratios between the concentration after and before ultracentrifugation. [e] Volume of spike solution added to 5 mL of plasma. [f] Dilution factor $(5 + V_{dil}/5)$. [g] Percentage bound to plasma proteins.

Table V—Red Blood Cell Partition Coefficients for III at Different Concentrations

A_{tot}, μg[a]	C_{PW}, ng/mL[b]	V_{PW}, mL[b]	Hematocrit	D[c]
0.050	7.7	3.15	0.370	1.82
0.050	7.6	3.08	0.385	1.84
0.175	26.3	3.15	0.370	1.89
0.175	24.3	3.05	0.390	2.14
0.500	73.1	3.18	0.365	2.00
0.500	78.0	3.00	0.400	1.70
1.750	284.7	3.70	0.260	1.88
1.750	261.0	3.00	0.400	1.85
5.000	783.0	3.03	0.395	1.70
5.000	821.6	3.05	0.390	1.56
17.500	2724.8	3.15	0.370	1.76
17.500	2651.5	2.95	0.410	1.78
Mean ± *SD*				1.83 ± 0.15

[a] Total amount of drug added to a 5.0-mL red blood cell suspension. [b] PW = plasma water. [c] Red blood cell-plasma water partition coefficient.

II and III. Protein binding of both compounds is low, averaging 30.1 ± 5.1% (*SD*) for II and 27.7 ± 2.5% (*SD*) for III, with no observable concentration dependence. The results are in excellent agreement with a study by Ludden *et al.* (12), who reported a concentration-independent protein binding of tritiated III in dog plasma of 26% over a concentration range of 1–500 ng/mL, determined by equilibrium dialysis. The protein binding of I had been determined to be concentration independent at 36% (13). The presence of I in up to 10-fold higher concentrations than its antagonists does not change the degree of plasma protein binding of II or III (Table IV).

Determination of the Red Blood Cell Partition Coefficient—The red blood cell partition coefficient (D) of a drug can be defined (11) as:

$$D = \frac{C_{RBC}}{C_{PW}} = \frac{A_{tot} - C_{PW}V_{PW}}{C_{PW}(V_B - V_{PW})} \quad \text{(Eq. 2)}$$

where C_{RBC} is the concentration of the drug in the erythrocyte, C_{PW} is the drug concentration in plasma water, A_{tot} is the total amount of drug added to the red blood cell suspension, and V_{PW} is the volume of plasma water (calculated from the hematocrit of the erythrocyte suspension).

The red blood cell partition coefficients of III were determined for six different concentrations (Table V), averaging 1.83 ± 0.15 (*SD*). There was no concentration dependence for the partitioning in the studied concentration range up to 3.5 μg/mL. This relatively high partition coefficient was confirmed by comparing the concentration of III in blood, C_B, and plasma, C_P, after spiking whole blood. The ratio of these two concentrations is related to the degree of protein binding in plasma, expressed as fraction bound (f_b), to the red blood cell partition coefficient D and the hematocrit (H) (14):

$$\frac{C_B}{C_P} = DH(1 - f_b) + 1 - H \quad \text{(Eq. 3)}$$

Substitution of the values previously obtained for D and f_b gives a blood/plasma ratio of 1.16 for blood with a hematocrit of 0.49. This calculated ratio agrees with that obtained experimentally (1.21 ± 0.08) in blood in the investigated concentration range.

The red blood cell partition coefficients of II in the same concentration range are given in Table VI, averaging 1.49 ± 0.25 (*SD*), also with no concentration dependence. Both partition coefficients for III and II were significantly higher than the D = 1.11 previously reported for I (13), in agreement with the hypothesis that erythrocyte partitioning is related to the lipophilicity, which is mirrored by the HPLC retention times on a reverse-phase column. This theory

Table VI—Red Blood Cell Partition Coefficients for II at Different Concentrations

A_{tot}, μg[a]	C_{PW}, ng/mL	D[b]
0.050	9.3	1.30
0.175	28.8	1.79
0.500	87.9	1.47
1.750	278.1	1.83
5.000	955.9	1.16
17.500	3166.7	1.41
Mean ± *SD*		1.49 ± 0.27

[a] Total amount of drug added to a 5.0-mL red blood cell suspension when the volume of plasma water (PW) was 3.45–3.70 mL and the hematocrits were 0.260–0.310. [b] Red blood cell-plasma water partition coefficient.

is further confirmed by the fact that the more lipophilic derivative of I, buprenorphine, has an extremely high red blood cell partition coefficient[17] of ~10.

No time dependence in the red blood cell partitioning could be observed for either compound. Samples assayed 4–5 min after spiking were not significantly different from the samples that were allowed to equilibrate for 60 min, indicating that the partitioning was relatively fast. The lack of correlation between the protein binding and red blood cell partitioning for the investigated compounds suggests that these two properties should be studied separately.

REFERENCES

(1) P. T. Kissinger, *Anal. Chem.*, **46,** 15R (1974).

(2) D. D. Koch and P. T. Kissinger, *J. Chromatogr.* **164,** 441 (1979).

(3) D. A. Roston and P. T. Kissinger, *Anal. Chem.*, **53,** 1695 (1981).

(4) J. E. Wallace, E. L. Shimek, S. Stavchansky, and S. C. Harris, *Anal. Chem.*, **53,** 960 (1981).

(5) S. H. Curry, E. A. Brown, O. Y.-P. Hu, and J. H. Perrin, *J. Chromatogr.*, **231,** 361 (1982).

(6) J. E. Wallace, S. G. Harris, and M. W. Peek, *Anal. Chem.*, **52,** 1328 (1980).

(7) M. W. White, *J. Chromatogr.*, **178,** 229 (1979).

(8) K. Ishikawa, J. L. Martinez, and J. L. McCaugh, *J. Chromatogr.*, **231,** 255 (1982).

(9) E. R. Garrett and T. Gürkan, *J. Pharm. Sci.*, **69,** 1116 (1980).

(10) "BAS 4A Manual," Bioanalytical Systems Inc., West Lafayette, Ind., 1981.

(11) H. Derendorf and E. R. Garrett, *J. Pharm. Sci.*, **72,** 630 (1983).

(12) T. M. Ludden, L. Malspeis, J. D. Baggot, T. D. Sokolski, S. G. Frank, and R. H. Reuning, *J. Pharm. Sci.*, **65,** 712 (1976).

(13) E. R. Garrett and A. J. Jackson, *J. Pharm. Sci.*, **68,** 753 (1979).

(14) E. R. Garrett and H. T. Lambert, *J. Pharm. Sci.*, **62,** 550 (1973).

(15) Stat 1-22A "Linear Regression" HP-65 Stat-Pac 1, Hewlett-Packard, Cupertino, Calif., pp. 49–51.

ACKNOWLEDGMENTS

Supported in part by Grant 2-RO1-DA-00743 from the National Institute of Drug Abuse, Rockville, MD 20852. Dr. A. A. El-Koussi was supported by Peace Fellowship Program for Egypt, administered by American Mid-East Educational and Training Services Inc., Washington, D.C.

[17] E. R. Garrett, V. Ravichandran, and H. Derendorf, unpublished results.