GLC Determinations of Plasma Acenocoumarol Levels

K. K. MIDHA ˣ **and J. K. COOPER**

Abstract □ A method for the quantitative estimation of acenocoumarol in plasma is described. Plasma containing acenocoumarol, to which a known amount of γ-oxo derivative of phenylbutazone is added as an internal standard, is acidified and extracted with ethylene dichloride. The drug and the internal standard are then back-extracted into alkali, which, in turn, is acidified and reextracted with ethylene dichloride. The organic extract is evaporated and treated with an ethereal solution of diazomethane (100 µl). The reacted mixture is evaporated, and the residue is dissolved in 25 µl of carbon disulfide. Aliquots (2–3 µl) are injected into a gas chromatograph equipped with a flame-ionization detector. The methyl derivatives of acenocoumarol and the internal standard give sharp, well-separated, symmetrical peaks. The method is of sufficient sensitivity to determine 0.25 µg/ml of the drug in plasma with a relative standard deviation of 4%.

Keyphrases □ Acenocoumarol—GLC analysis, human and dog plasma □ GLC—analysis, acenocoumarol, human and dog plasma □ Anticoagulants—acenocoumarol, GLC analysis, human and dog plasma

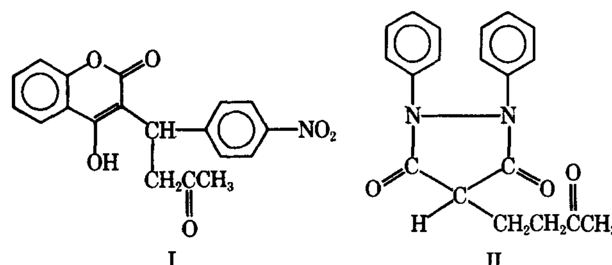
Acenocoumarol (I) is an oral anticoagulant with an onset of action similar to that of dicumarol. It is structurally related to warfarin, both drugs belonging to the coumarin class. Although GLC procedures for the quantitative determination of warfarin (1, 2) and phenprocoumon (3) and a high-pressure liquid chromatographic (HPLC) procedure for quantitating warfarin (4) from plasma have been reported, no GLC or HPLC procedures for quantitating acenocoumarol from biological fluids have been described.

The chromatographic behavior of I and its trimethylsilyl ether derivative was investigated using an aluminum column packed with silicone gum rubber on dimethyldichlorosilane-treated, flux-calcined diatomite support (5). However, this procedure was not applied to plasma level determinations. To facilitate study of the pharmacokinetics and potential interactions after single- and multiple-dose oral administration of I, a sensitive and apparently specific GLC procedure was developed for the measurement of the intact drug in plasma.

EXPERIMENTAL

Reagents—Ethylene dichloride[1] and ether[2] were glass distilled prior to use. Stock solutions containing 100 µg of I[3]/ml were prepared daily in 0.1 N NaOH and stored in the dark at 4°. Appropriate dilutions (0.25–4.0 µg/ml) were made immediately before use. Aqueous solutions containing 100 µg/ml of the internal standard, the γ-oxo derivative of phenylbutazone[4] (II), were prepared weekly in 0.1 N NaOH and diluted daily to 15 µg/ml with distilled water. An ethereal solution of diazomethane was synthesized from N-methyl-N-nitroso-p-toluenesulfonamide[5].

Plasma Level Study—Acenocoumarol was administered orally in gelatin capsules to two dogs. One dog, 35 kg, received 1 mg/kg of I; a second dog, 30 kg, was given 0.5 mg/kg of I. Blood samples (10 ml) were withdrawn from the cephalic vein of each dog, using heparinized evacuated tubes[6], at 14 appropriate time intervals for 96 hr. The blood samples



were centrifuged, and the plasma was transferred to another tube before storage at −15°.

Extraction of Acenocoumarol—To a 2-ml human or canine plasma sample ("spiked" or from dosed animals) in a glass-stoppered centrifuge tube[7] (50 ml) were added 1 ml of the internal standard solution (II, 15 µg), 3 ml of 3 N HCl, and 20 ml of ethylene dichloride. The samples were stoppered and extracted by shaking at an angle of 30° for 30 min at 180 cpm on a flat-bed shaker[8]. After centrifugation at 3000 rpm for 10 min, the aqueous layer was removed by aspiration; 18 ml of the organic phase was then transferred into another glass-stoppered centrifuge tube[7] (50 ml) containing 10 ml of 1 N NaOH.

The extraction was repeated (shaking for 15 min and centrifuging for 10 min), and 9 ml of the aqueous phase was transferred to another glass-stoppered tube[7] (50 ml) containing 3 ml of 5 N HCl. The tube was

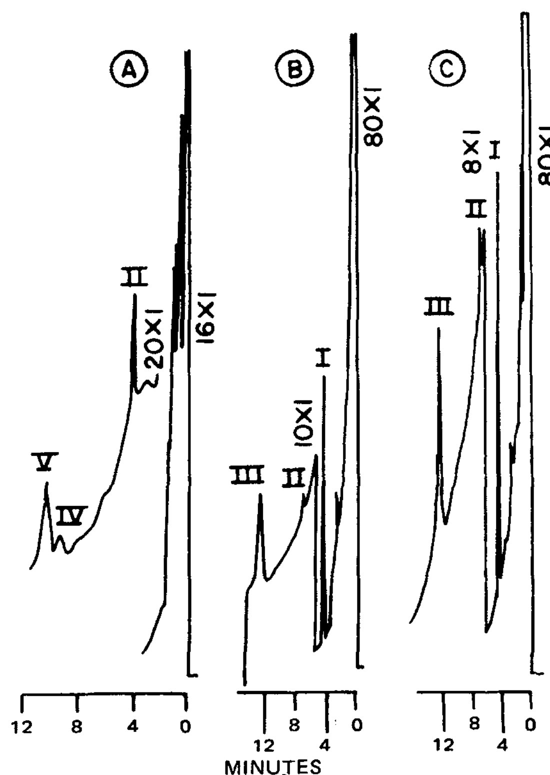
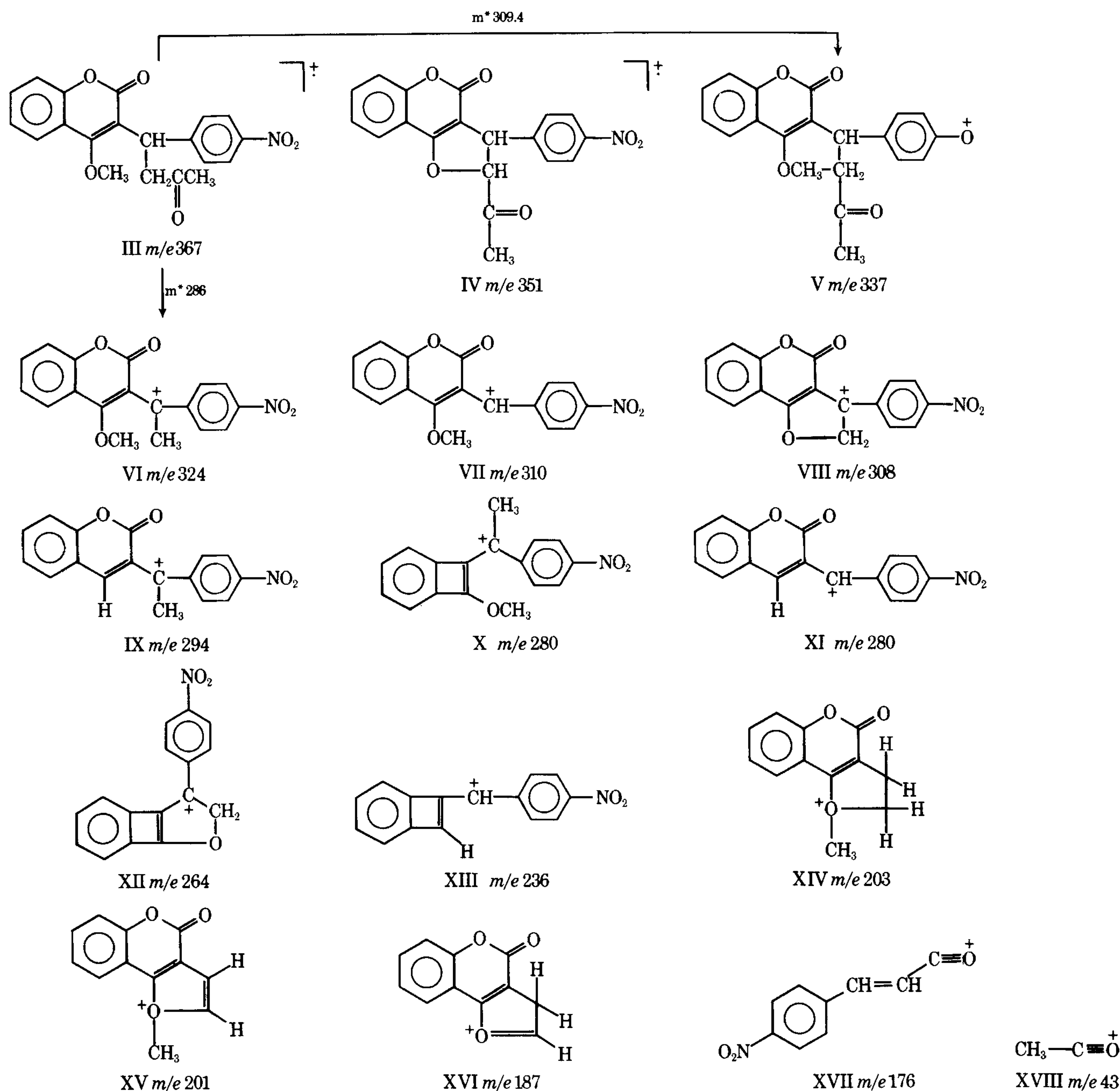


Figure 1—*Gas chromatograms of human plasma. Key: A, control plasma; B, plasma containing 1.0 µg/ml of I and 7.5 µg/ml of II; and C, plasma (7 hr) from a dog given 1 mg of I/kg po containing I (1.24 µg/ml) and II (7.5 µg/ml). Peak I is methylated II, and peak III is methylated I. Peaks II, IV, and V are due to endogenous materials in plasma.*

[1] Caledon Laboratories Ltd., Georgetown, Ontario, Canada.
[2] Mallinckrodt Chemical Works Ltd., Montreal, Quebec, Canada.
[3] Geigy Pharmaceuticals, Dorval, Quebec, Canada.
[4] United Pharmaceutical Works, Praha, Czechoslovakia.
[5] Diazald, Aldrich Chemical Co., Milwaukee, Wis.
[6] Vacutainers, Becton-Dickinson Co., Mississauga, Ontario, Canada.
[7] Fisher Scientific Co., Montreal, Quebec, Canada.
[8] Eberbach Corp., Ann Arbor, Mich.



Scheme I

swirled gently, and the extraction was carried out with another 20 ml of ethylene dichloride for 20 min. The aqueous layer was removed by aspiration, and 18 ml of the organic phase was transferred to a glass-stoppered centrifuge tube[7] (50 ml).

The organic extract was evaporated[9] at 85° under a stream of dry nitrogen to a volume of approximately 4 ml. A crystal of anhydrous calcium chloride[7] was added, and the tube was swirled gently. The extract was transferred quantitatively into a custom-made evaporating tube (6) containing a small antibumping granule[10] and evaporated as before to dryness.

The tube was then allowed to cool to room temperature, and the residue was dissolved in 100 µl of ethereal diazomethane by mixing[11] for 30 sec. After the solution was left to incubate for 10 min, it was evaporated to dryness at room temperature. The evaporated extract was dissolved in 25 µl of carbon disulfide by mixing[11] for 30 sec, and aliquots (2–3 µl) were then injected into the gas chromatograph.

GLC—A gas chromatograph[12] equipped with a flame-ionization detector was employed. The column was a spiral glass tube[12], 180 × 0.25 cm i.d., packed with 3% phenyl methyl dimethyl silicone[13] coated on acid-washed, dimethylchlorosilane-treated, 80–100-mesh, high performance, flux-calcined diatomite support[14].

The column was conditioned by maintaining the column at 340° for 18 hr with a low nitrogen flow. In use, the injection port, detector, and column oven temperatures were 300, 310, and 285°, respectively. The nitrogen carrier gas was maintained at a flow rate of 70 ml/min. Hydrogen and compressed air flow rates were adjusted to give maximum response.

Calculations—Peak height ratios were calculated by dividing the height of the peak due to methylated I (7.1 min) by that of methylated II (2.6 min). Calibration curves were constructed from the results of

[9] Thermolyne Dri-Bath, Fisher Scientific Co., Montreal, Quebec, Canada.
[10] British Drug House, Toronto, Ontario, Canada.
[11] Vortex Genie, Fisher Scientific Co., Montreal, Quebec, Canada.
[12] Model 3920, Perkin-Elmer, Montreal, Quebec, Canada.
[13] OV-11, Chromatographic Specialties, Brockville, Ontario, Canada.
[14] Chromosorb W, Chromatographic Specialties, Brockville, Ontario, Canada.

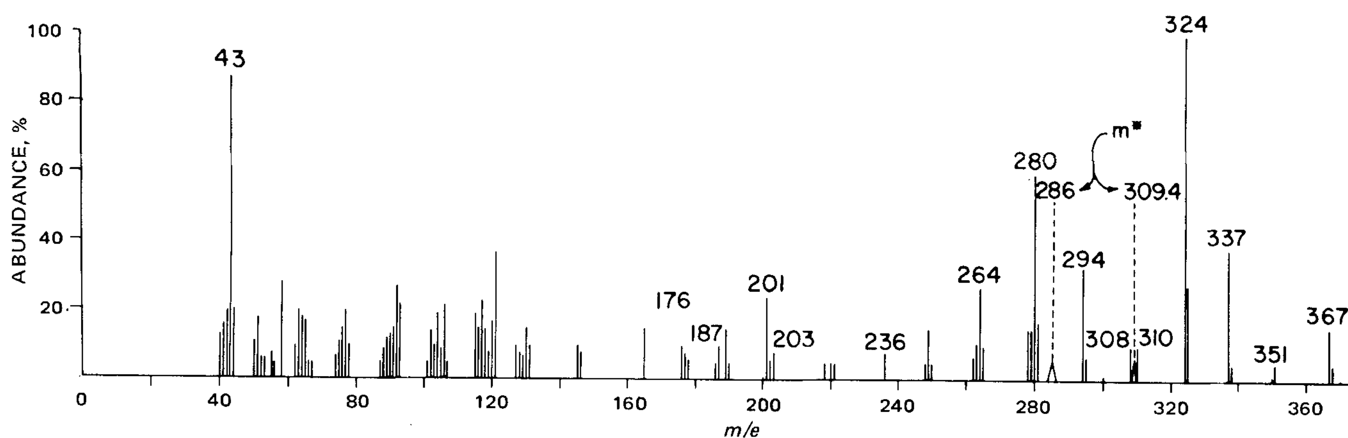


Figure 2—*GLC–mass spectrum (normalized) of methylated acenocoumarol.*

spiked control plasma samples by plotting the peak height ratios against the concentration of I (micrograms per milliliter of plasma).

RESULTS AND DISCUSSION

Methylation of I with diazomethane gave one peak on GLC analysis with a retention time of 7.1 min (peak III, Fig. 1B). Methylation of II with diazomethane also gave one peak on GLC with a retention time of 2.6 min (peak I, Fig. 1B). Combined GLC–mass spectrometric[15] analysis was carried out on the compound giving rise to peak III (Fig. 1B).

The mass spectrum (Fig. 2) showed a molecular ion at m/e 367 and abundant ions at m/e 351, 337, 324, 310, 308, 294, 280, 264, 236, 203, 201, 187, 176, and 43. Structures III–XVIII (Scheme I) are postulated for these ions. These fragmentations are in agreement with those reported for warfarin (2, 7) and phenprocoumon (3, 8, 9) and suggest that the methylated derivative of I has the structure 3-(α-acetonyl-p-nitrobenzyl)-4-methoxycoumarin. Combined GLC–mass spectral evidence for the structure of the internal standard (peak I, Fig. 1B, retention time of 2.6 min) indicated it to be 1,2-diphenyl-4-methyl-4-(2-butanone)-3,5-dioxopyrazoline (molecular ion at m/e 336 and other diagnostic ions at m/e 321, 308, 293, 279, 264, 202, 177, 160, 119, 77, 51, and 43).

Attempts to prepare a methyl derivative of I by the safer flash-heater methylation technique using trimethylanilinium hydroxide were unsuccessful. However, ethereal diazomethane was effective in the methylation of I and other coumarin derivatives such as phenprocoumon[16] and warfarin[17], the later two being investigated as possible internal standards for the assay of I. These derivatives gave sharp well-defined GLC peaks; however, phenprocoumon and warfarin could not be employed as internal standards because their methyl derivatives eluted too close to the solvent front. Since methylated II [a metabolite of phenylbutazone in humans (10)] gave a single GLC peak with the desired retention time and had similar partition characteristics to I, it was chosen as an internal standard for the assay of I.

Several other liquid phases such as saturated hydrocarbon lubricant[18], methyl silicone gum[19], phenyl methyl silicone fluid[20], and phenyl methyl dimethyl silicone fluid[21] were tested, but the peak for methylated I under these conditions was either not sharp or had interference from plasma constituents. When carbon disulfide was employed as the injection solvent, it rendered a very small solvent peak. Untreated plasma extracts were much less stable than those reacted immediately after extraction. The plasma extracts that had been reacted with diazomethane were stable up to 12 hr at room temperature.

Figure 1A shows a typical chromatogram obtained by processing fresh blank plasma from humans as described under *Experimental.* Extraneous peaks II, IV, and V were observed in chromatograms of all human plasma samples, but IV and V were not present in chromatograms of dog plasma samples. A chromatogram obtained when the method was applied to spiked plasma containing 1.0 μg of I/ml and 7.5 μg of II/ml is shown in Fig. 1B. Extraneous peaks II, IV, and V did not interfere with the peak from the internal standard (peak I, retention time of 2.6 min) or acenocoumarol (peak III, retention time of 7.1 min). Figure 1C shows a chromatogram obtained from a 7-hr sample (1 ml) of a 35-kg dog which received an extemporaneously prepared gelatin capsule containing 35 mg of I.

The overall recoveries of I and II from plasma were 99.90 ± 1.25 and 47.42 ± 1.64%, respectively (Table I). The accuracy and precision of the GLC assay are demonstrated in Table II. Results are based on at least five determinations of each acenocoumarol concentration (0.25–4.0 μg/ml), treated as described under *Experimental.* The overall relative standard deviation was 3.12%. The calibration curve obtained by plotting the peak height ratio of methylated I/II *versus* the concentration of I was linear ($y = mx$) over the concentration range of 0.25–4.0 μg of I/ml of plasma. A mean slope value of 0.0492 ± 0.0006 ($p = 0.05$; $r^2 = 0.998$) was obtained. The peak height ratio of the drug and the internal standard was used as the index of detector performance and overall efficiency of the analytical procedure.

The application of the method to plasma level determinations in dogs is demonstrated in Fig. 3. This figure shows the plasma profiles over 96 hr for Dogs A (35 kg) and B (30 kg), which received 1 and 0.5 mg/kg, respectively, of I orally in extemporaneously prepared capsules. The specificity of the analytical procedure was determined by pooling the remaining plasma from dosed dogs, extracting and derivatizing it as described under *Experimental.* A GLC–mass spectrum of the compound giving rise to the resultant peak for I was obtained and compared with that of authentic methylated I. No ions other than those for methylated I were observed in the mass spectrum of the peak from pooled plasma samples, indicating virtually no interference.

The described GLC procedure is sensitive for the determination of acenocoumarol, and large plasma samples are not required. It also offers

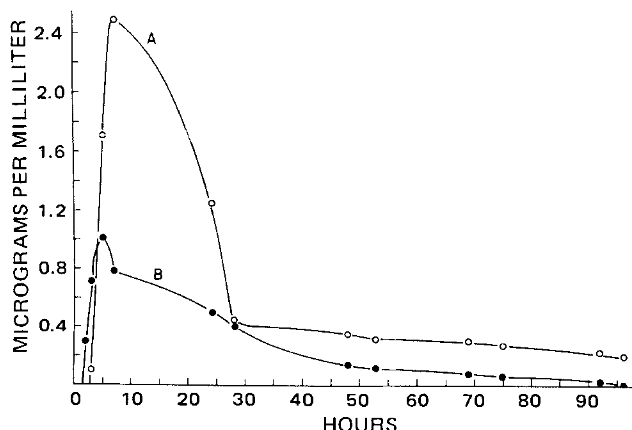


Figure 3—*Plasma profiles of acenocoumarol. Key: Dog A (35 kg), 1.0 mg/kg po; and Dog B (30 kg), 0.5 mg/kg po.*

[15] Perkin-Elmer model 900 gas chromatograph attached to a Hitachi Perkin-Elmer model RMSU mass spectrometer through a jet separator.
[16] Marcumar, Hoffmann-La Roche Inc., Montreal, Quebec, Canada.
[17] Warfarin sodium, Warner-Lambert, Scarborough, Ontario, Canada.
[18] Apiezon-L, Chromatographic Specialties, Brockville, Ontario, Canada.
[19] SE-30, Chromatographic Specialties, Brockville, Ontario, Canada.
[20] OV-17, Chromatographic Specialties, Brockville, Ontario, Canada.
[21] OV-7, Chromatographic Specialties, Brockville, Ontario, Canada.

Table I—Recovery of I and II from Plasma (n = 4)

Compound	Micrograms Added to 1 ml of Plasma	Mean Micrograms Recovered	Mean Percent Recovery	*SD* of Percent Recovery
I	1.00	0.996	99.60	1.11
	2.00	2.04	100.20	1.37
	Mean = 99.90 ± 1.25%			
II	1.00	0.47	47.39	0.01
	2.00	0.95	47.46	0.01
	Mean = 47.42 ± 1.64%			

Table II—GLC Estimation of I Added to Plasma

Added I, µg	n	Mean Peak Height Ratio I/II	*SD*	*RSD*
0.25	7	0.012	0.001	3.98
0.50	6	0.024	0.001	3.13
1.00	7	0.048	0.002	3.19
2.00	6	0.095	0.002	1.76
4.00	5	0.200	0.001	4.14
			Mean *RSD*	3.12
$y = mx$, where $m = 0.0492 \pm 0.0006$; $r^2 = 0.998$				

the advantage that the intact drug is measured. The technique is being used for investigating single- and multiple-dose pharmacokinetics.

REFERENCES

(1) D. G. Kaiser and R. S. Martin, *J. Pharm. Sci.*, **63**, 1579 (1974).

(2) K. K. Midha, I. J. McGilveray, and J. K. Cooper, *ibid.*, **63**, 1725 (1974).

(3) K. K. Midha, J. W. Hubbard, J. K. Cooper, and I. J. McGilveray, *ibid.*, **65**, 387 (1976).

(4) E. S. Vessell and C. A. Shively, *Science*, **184**, 466 (1974).

(5) F. W. Deckert, *J. Chromatogr.*, **69**, 201 (1972).

(6) A. H. Beckett, *Dan. Tidssk. Farm.*, **40**, 197 (1966).

(7) W. F. Trager, R. J. Lewis, and W. A. Garland, *J. Med. Chem.*, **13**, 1196 (1970).

(8) L. R. Pohl, R. Haddock, W. A. Garland, and W. F. Trager, *ibid.*, **18**, 513 (1975).

(9) R. E. Haddock, W. F. Trager, and L. R. Pohl, *ibid.*, **18**, 519 (1975).

(10) I. J. McGilveray, K. K. Midha, and N. Mousseau, *Pharmacologist*, **16**, 218 (1974).

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Binding of Codeine, Morphine, and Methadone to Human Serum Proteins

JOSEPH JUDIS

Abstract □ The binding properties of codeine, morphine (as representative opium alkaloids), and methadone (a synthetic pharmacologically similar compound) were studied with selected human serum proteins. The methodology involved equilibrium and dynamic dialysis using ^{3}H- and/or ^{14}C-labeled compounds. For estimation of the percent binding with equilibrium dialysis, concentrations of the ligand used were approximately therapeutic blood levels and another concentration 30–60 times higher. The percent binding to whole human serum ranged from about 20% for morphine to almost 60% for methadone. Of the human serum proteins investigated, the highest percent binding was found with albumin, except for methadone for which it was β-globulin III. The affinity for other serum proteins varied with the ligand. In studies with albumin using dynamic dialysis, the plots of nubar divided by free concentration *versus* nubar were similar for all three ligands studied and had positive slopes, unlike those reported for acidic compounds for which the slope is always negative. In studies of binding of one ligand in the presence of another, significant competition was demonstrated, suggesting that the same binding sites were involved.

Keyphrases □ Codeine—binding to human serum proteins □ Morphine—binding to human serum proteins □ Methadone—binding to human serum proteins □ Binding, protein—codeine, morphine, and methadone to human serum □ Protein binding—codeine, morphine, and methadone to human serum □ Analgesics, narcotic—codeine, morphine, and methadone, binding to human serum proteins □ Alkaloids, opium—codeine, morphine, and methadone, binding to human serum proteins

The binding of drugs to serum proteins is well established as a parameter in pharmacological and therapeutic activities of medicinal agents (1). Several reviews treated the methodology for studying protein binding and summarized the vast number of substances already investigated (2–5). Of the hundreds of compounds studied, the vast majority are acidic or nonpolar; few studies have

Table I—Binding of Methadone, Morphine, and Codeine to Various Human Serum Proteins[a]

Serum Protein Fraction	Concentration, mg/ml	Methadone 9.3×10^{-8} Mole	Methadone 3.3×10^{-6} Mole	Morphine 5.5×10^{-8} Mole	Morphine 3.6×10^{-6} Mole	Codeine 5.6×10^{-8} Mole	Codeine 3.4×10^{-6} Mole
		Ligand, % Bound for Concentration Indicated					
Albumin	40.0	31.16	21.84	23.24	16.56	14.26	28.50
		(2.23)	(0.29)	(1.30)	(1.15)	(0.68)	(0.09)
α-Globulin IV-1	1.0	10.19	5.70	1.61	4.10	9.59	5.03
		(1.28)	(0.51)	(0.07)	(0.98)	(1.46)	(0.21)
α-Globulin IV-4	5.0	12.53	4.58	6.42	7.56	12.34	17.05
		(1.65)	(0.13)	(0.01)	(0.39)	(0.70)	(0.40)
β-Globulin III	7.0	37.75	10.90	7.39	3.16	7.98	3.68
		(2.76)	(0.56)	(0.05)	(0.21)	(0.60)	(1.04)
γ-Globulin II	11.0	8.26	6.47	4.16	10.02	5.71	8.60
		(0.72)	(0.40)	(0.20)	(2.72)	(0.72)	(1.16)
Human serum	—	59.78	39.54	24.02	20.08	29.01	22.35
		(3.00)	(1.20)	(1.82)	(1.04)	(2.70)	(0.87)

[a] The number of moles of ligand added to each system is indicated as the concentration. Values in parentheses are standard deviations.