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Binding of Metronidazole and Its Derivatives to Plasma Proteins: An Assessment of Drug Binding Phenomenon

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Abstract □ Metronidazole and four derivatives were studied *in vitro* to investigate the differences in the extent of their binding to plasma proteins. Modification at the terminal portion of the alkyl side chain resulted in wide differences in the extent of binding. Molecular orbital calculations were performed by the CNDO and MINDO/2 methods to estimate the frontier electron density on the hetero atom at the 3′-position of the alkyl side chain. A linear correlation between the protein binding parameter ($\log_e P$) and the frontier electron density (q_r) was observed for the binding of this group of trichomonicidal drugs. NMR spectroscopy was used to demonstrate that the alkyl side chain participated in the binding of these compounds to plasma proteins.

Keyphrases □ Metronidazole and four derivatives—plasma protein binding, correlation with frontier electron density □ Plasma protein binding—metronidazole and four derivatives, correlation with frontier electron density □ Electron density, frontier—metronidazole and four derivatives, correlation with plasma protein binding parameters

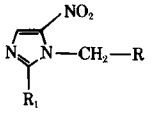
Several reports provided information on the theoretical, biological, and clinical aspects of drug–protein binding phenomena (1–6). The majority of these investigations was concerned with the drug–serum albumin interaction, since it is the predominant phenomenon occurring in humans. Quantitative correlation has been found between physicochemical parameters such as lipophilicity, pKa, and electronic charge density and the strength of drug binding to serum albumin (7–10).

It is well recognized that the therapeutic activity of a drug is primarily dependent upon the availability of its free (unbound) permeable species at the effective receptor sites located either in the vascular or extravascular compartments of the body (1, 11–14). Recent investigations demonstrated that any structural modification in a functional group can influence significantly both the activity and the extent of protein binding of disopyramide phosphate and its derivatives (14–16).

Metronidazole[1] has been shown to be an effective antiprotozoal agent with a broad spectrum of activity against anaerobes (17–21). In this study, it was observed that the extent of plasma protein binding of

[1] Flagyl, G. D. Searle & Co., Chicago, IL 60648

Table I—Structural Formulas and Other Parameters for Metronidazole and Its Derivatives



Compound	R	R_1	Partition[a] Coefficient
I	$—CH_2OH$	CH_3	0.778 ± 0.029
II	$—CH_2SSO_3Na$	CH_3	0.027 ± 0.01
III	$—CH_2NHCOCH_3$	CH_3	0.339 ± 0.008
IV	—COOH	H	0.030 ± 0.001
V	$—CH_2OCOCH_3$	CH_3	2.00 ± 0.07

[a] Determined in the system of *n*-octanol–pH 7.4 phosphate buffer.

Table II—Binding of Metronidazole and Its Derivatives to Human Plasma Proteins

Total Drug Concentration, $M \times 10^5$	Fraction of Drug Bound, %				
	Metronidazole Derivatives				
	I	II	III	IV	V
40.0	0.43	—	—	4.45	—
24.0	1.06	—	—	2.72	—
8.0	—	—	—	—	1.51
6.4	3.07	66.37	11.0	—	2.67
4.8	4.22	67.12	11.36	4.45	2.36
3.2	3.74	68.33	10.85	1.86	1.36
1.6	—	71.18	—	5.93	2.86

metronidazole and its derivatives is significantly dependent upon the nature of their alkyl side chains. Molecular conformation analysis, coupled with frontier electron density (q_r) calculations by the CNDO[2] and MINDO/2[3] methods, was performed to support and rationalize these observations. NMR spectroscopy was also used to confirm the participation of the alkyl side chain in the binding process. This article reports the results and interpretations of these investigations.

EXPERIMENTAL

Materials—The following were used: metronidazole[4] (I), 1β-thiosulfate sodium methyl-2-methyl-5-nitroimidazole[5] (II), 1β-aminoacetylethyl-2-methyl-5-nitroimidazole[6] (III), 1β-carboxymethyl-5-nitroimidazole[7] (IV), and metronidazole acetate (V). All compounds were tested for structure identity and purity prior to use.

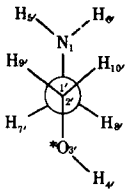
Plasma Protein Binding Study—The procedure reported previously (16) was utilized.

CNDO and MINDO/2 Study—These studies were conducted on the basis of established procedures (22–24). All calculations were performed with a high-speed memory computer[8].

NMR Study—Lyophilized normal human serum[9], 40% NaOD in D_2O[10], and 38% DCl in D_2O[11] were used to prepare appropriate stock solutions in 99.7% pure D_2O[11]. An appropriate amount of the drug was accurately weighed and transferred to a 10-ml volumetric flask, followed by the addition of 3–6 ml of D_2O to dissolve the drug. The pD of the solution was adjusted to a near neutral region (6–8) with the addition of a few drops of dilute NaOD or DCl solution.

Aliquots of a freshly prepared stock solution of human serum (containing 4.6% serum albumin) in D_2O were then added to the solution and the pD was readjusted to 7. The final volume of the solution was made up with D_2O to obtain a 0.0467 *M* solution of the drug without albumin and with human serum containing 1.15 and 2.5% albumin. All NMR spectra were obtained on a high-resolution (60 MHz) NMR spectrometer[12]. A 1% tetramethylsilane[11] solution in $CDCl_3$ was used as an external standard to calibrate the instrument for all measurements.

Table III—Coordinate System and Minimized Energy of $H_2NCH_2CH_2OH$ in Bohr Units[a]



Atom	*x*	*y*	*z*
N_1	0.02975	−0.05078	0
C_1'	2.72334	0.13006	0
C_2'	3.54814	2.88035	0
$*O_3'$	6.02929	3.22673	0
H_4'	7.98232	3.94783	0
H_5'	−1.26634	1.61069	0
H_6'	−0.95916	−1.90575	0
H_7'	3.47664	−1.09985	1.80593
H_8'	3.47664	−1.09985	−1.80593
H_9'	2.42889	3.94561	1.78834
H_{10}'	2.42889	3.94561	−1.78834

[a] Total energy = −32.64253 a.u. (MINDO/2). Frontier electron density at 3′-position = 0.1255 (CNDO).

RESULTS AND DISCUSSION

Table I illustrates the structural formulas and the effect of alkyl side-chain modification on the lipophilic character of metronidazole and its derivatives. In these five nitroimidazole derivatives, the functional group on the *N*-1 position of the imidazole nucleus was varied chemically to assess its effects on the extent of their

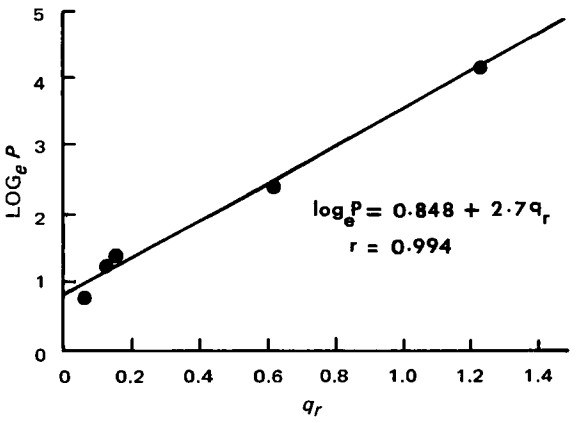


Figure 1—*Linear relationship between the protein binding parameter ($\log_e$ P) and frontier electron density (q_r) for I and its derivatives.*

[2] QCPE-141 computer program by P. Dobosh, Carnegie-Mellon University, Pittsburgh, Pa.

[3] QCPE-217 computer program by A. Komornicki and J. McIver, Department of Chemistry, State University of New York, Buffalo, N.Y.

[4] SC-10295; marketed as Flagyl by G. D. Searle.

[5] SC-21253.

[6] SC-24231.

[7] SC-28905.

[8] A 600/6000 series, Honeywell Corp., Minneapolis, Minn.

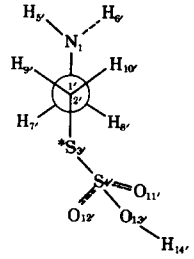
[9] Metrix, Lot 5466, Armour Pharmaceutical Co., Chicago, Ill.

[10] Aldrich Chemical Co., Milwaukee, Wis.

[11] Merck & Co., Rahway, N.J.

[12] Model A60-D, Varian Associates, Palo Alto, Calif.

Table IV—Coordinate System and Minimized Energy of $H_2NCH_2CH_2S_2O_3H$ in Bohr Units[a]



Atom	x	y	z
N_1	0	0	0
C_1'	2.64567	0	0
C_2'	3.61712	2.74331	0
$*S_3'$	6.45177	2.74331	0
S_4'	6.51443	6.33331	0
H_5'	−1.00157	1.73478	0
H_6'	−1.00157	−1.73478	0
H_7'	3.33956	−0.97975	1.69698
H_8'	3.33956	−0.97975	−1.69698
H_9'	2.92519	3.72445	1.69698
H_{10}'	2.92519	3.72445	−1.69698
O_{11}'	5.12476	7.35440	−2.43748
O_{12}'	5.12476	7.35440	2.43748
O_{13}'	9.38180	7.29272	0
H_{14}'	9.43127	9.18183	0

[a] Total energy = −108.48450 a.u. (CNDO). Frontier electron density at the 3'(*)-position = 1.2487 (CNDO).

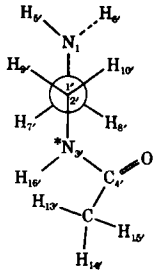
binding to plasma proteins (Table II). The concentration range studied for each drug was based on realistic values equivalent to or above the minimum inhibitory concentration (MIC) required against *Trichomonas vaginalis*.

The extent of plasma protein binding (Table II) and the lipophilic character (Table I) of this group of compounds do not show any linear correlation. This finding is demonstrated by the fact that metronidazole acetate (V), despite its high lipophilicity, shows poor protein binding (Tables I and II), whereas II, with poor lipophilic character, exhibits extensive binding to plasma proteins. Thus, a substitution of the hydroxy group with a thiosulfonyl group on II resulted in an approximately 16-fold increase in the binding of II over metronidazole. On the other hand, substitution with an acetamido group in III resulted in a slight increase in the extent of binding of that compound, despite a 10-fold increase in its lipophilicity over that of II.

CNDO and MINDO/2 calculations were performed to investigate conformational similarity and to assess whether the frontier electron density (q_r) on the hetero-atom at the terminal portion of the alkyl side chain contributed to these observed differences in the extent of protein binding. It is reasonable to assume that the contributions of the nitroimidazole moiety common to all these compounds is approximately the same with reference to the plasma protein binding phenomenon. This assumption implies that the observed differences in the protein binding of these compounds are due only to the variation of the functional group on the *N*-1 position of the imidazole ring. Under this condition, electron densities on the terminal molecular groups, namely H_2N—CH_2CH_2OH, H_2N—$CH_2CH_2SSO_3H$, H_2N—$CH_2CH_2NHCOCH_3$, H_2N—CH_2COOH, and H_2N—$CH_2CH_2OCOCH_3$, were computed on the basis that these parts of the molecule form localized molecular orbitals. Consequently, certain physicochemical properties of these functional groups can be considered nearly independent of their associated nitroimidazole nucleus.

The computations were performed in the following manner. At first, molecular conformation was roughly optimized by the CNDO (Complete Neglect of Differential Overlap) method (22). Second, the molecular energy was extensively minimized by the MINDO/2 (Modified Intermediate Neglect of Differential Overlap) method (23). These two methods belong to the classical semiempirical

Table V—Coordinate System and Minimized Energy of $H_2NCH_2NHCOCH_3$ in Bohr Units[a]



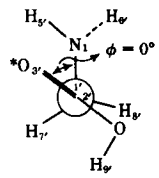
Atom	x	y	z
N_1	0.072210	0.01824	0.00000
C_1'	2.77920	0.059677	0.00000
C_2'	3.80939	2.718158	0.00000
$*N_3'$	6.55198	2.86855	−0.00000
C_4'	7.86141	5.17065	0.00000
H_5'	−1.07487	1.79479	0.00000
H_6'	−1.03062	−1.78361	−0.00000
H_7'	3.53119	−1.11542	−1.85245
H_8'	3.53119	−1.115421	1.85245
H_9'	2.98725	3.82200	−1.85647
H_{10}'	2.98726	3.82200	−1.856471
O_{11}'	6.71896	7.14799	−0.00000
C_{12}'	10.65700	5.12682	−0.00000
H_{13}'	11.62171	7.21036	0.00000
H_{14}'	11.58767	4.08591	−1.81875
H_{15}'	11.58767	4.08591	1.81875
H_{16}'	7.5661	0.99440	0.00000

[a] Total energy = −51.36125 a.u. (MINDO/2). Frontier electron density at the 3'(*)-position = −0.6374 (CNDO).

SCF-MO (Self-Consistent Field Molecular Orbital) method. Although both methods are not quantitatively as accurate as *ab initio* SCF-MO calculations (24), they reflect a quantitative estimation of physicochemical properties, such as frontier electron density of relatively large molecules.

Whereas the MINDO/2 method provides satisfactory results in terms of molecular conformation, the CNDO method provides computation of the frontier electron density (q_r) because its differ-

Table VI—Coordinate System and Minimized Energy of H_2NCH_2COOH in Bohr Units[a]



Atom	x	y	z
N_1	−0.04768	−0.08112	0
C_1'	2.62060	0.30440	0
C_2'	3.35046	3.05365	0
$*O_3'$	1.68097	4.66750	0
O_4'	5.77752	3.51890	0
H_5'	7.60299	4.54614	0
H_6'	−1.48876	1.45411	0
H_7'	−0.89224	−2.00850	0
H_8'	3.49451	−0.87853	1.78392
H_9'	3.49451	−0.87853	−1.78392

[a] Total energy = −42.98751 a.u. (MINDO/2). Frontier electron density at the 3'(*)-position = 0.1554 (CNDO).

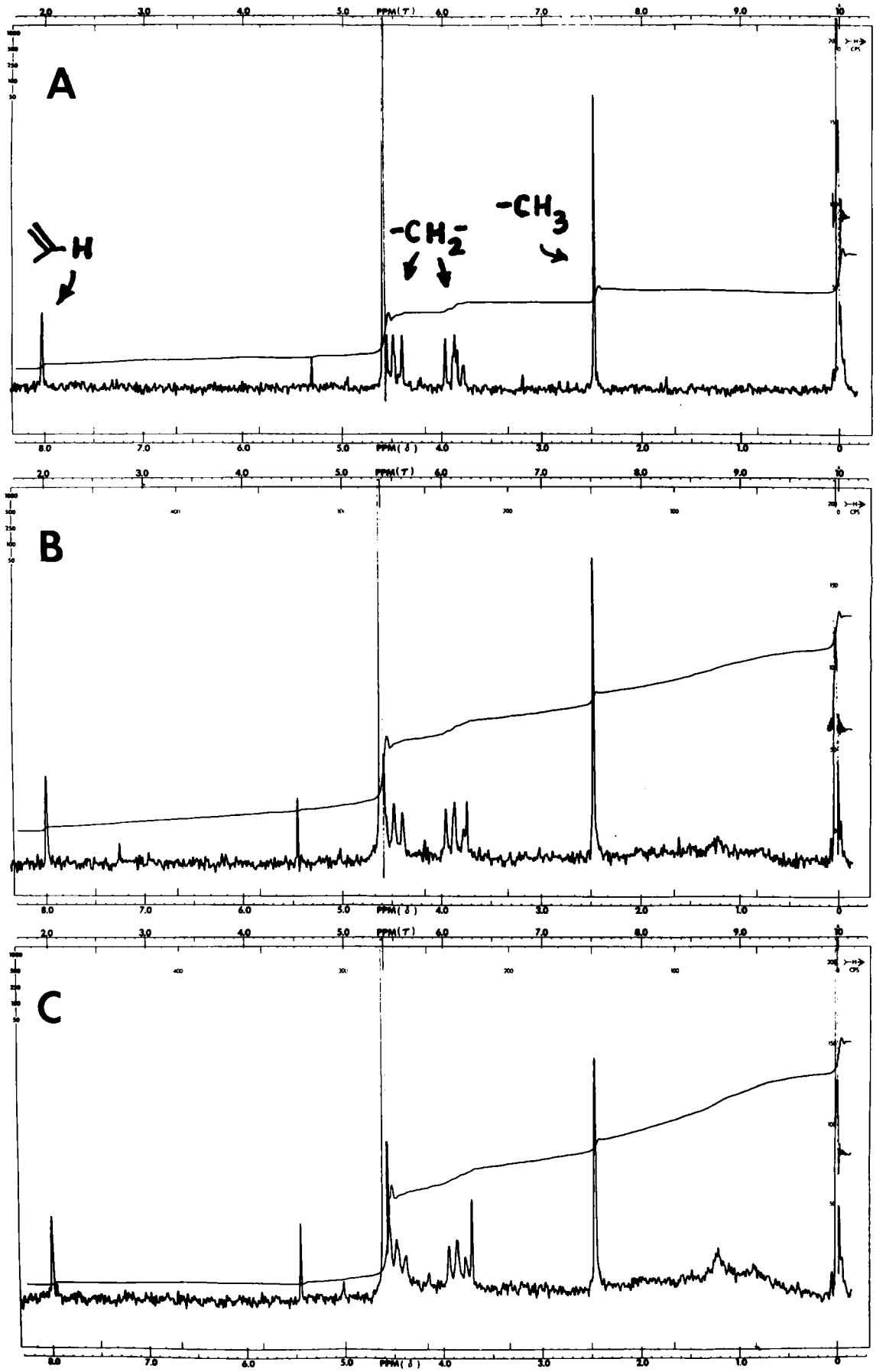


Figure 2—*NMR spectra illustrating the effect of human serum albumin on the alkyl protons of I at pD 7. Key: A, no protein; B, with 1.15% human serum albumin; and C, with 2.3% human serum albumin.*

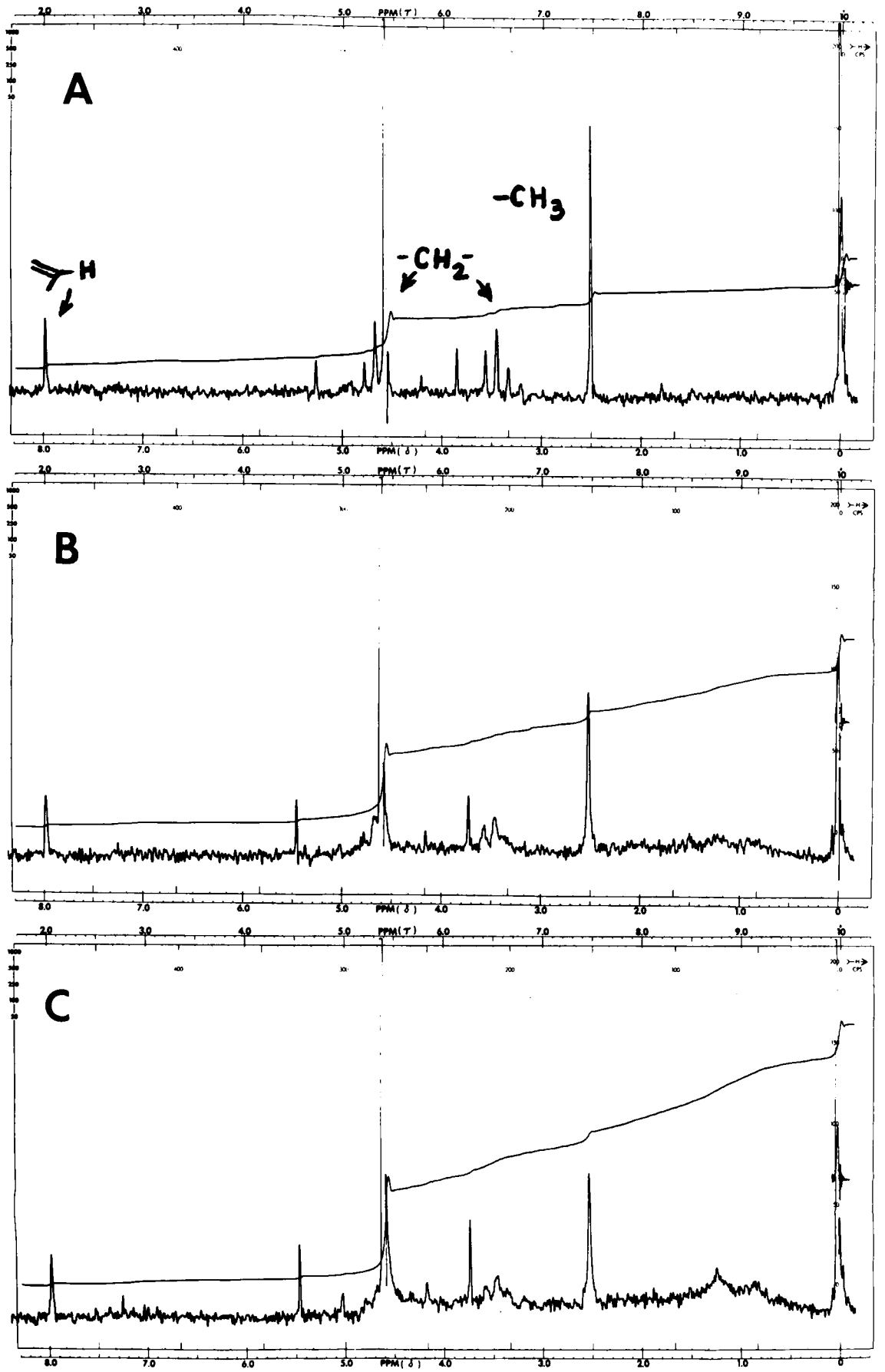


Figure 3—*NMR spectra illustrating the effect of human serum albumin on the alkyl protons of II at pD 7. Key: A, no protein; B, with 1.15% human serum albumin; and C, with 2.3% human serum albumin.*

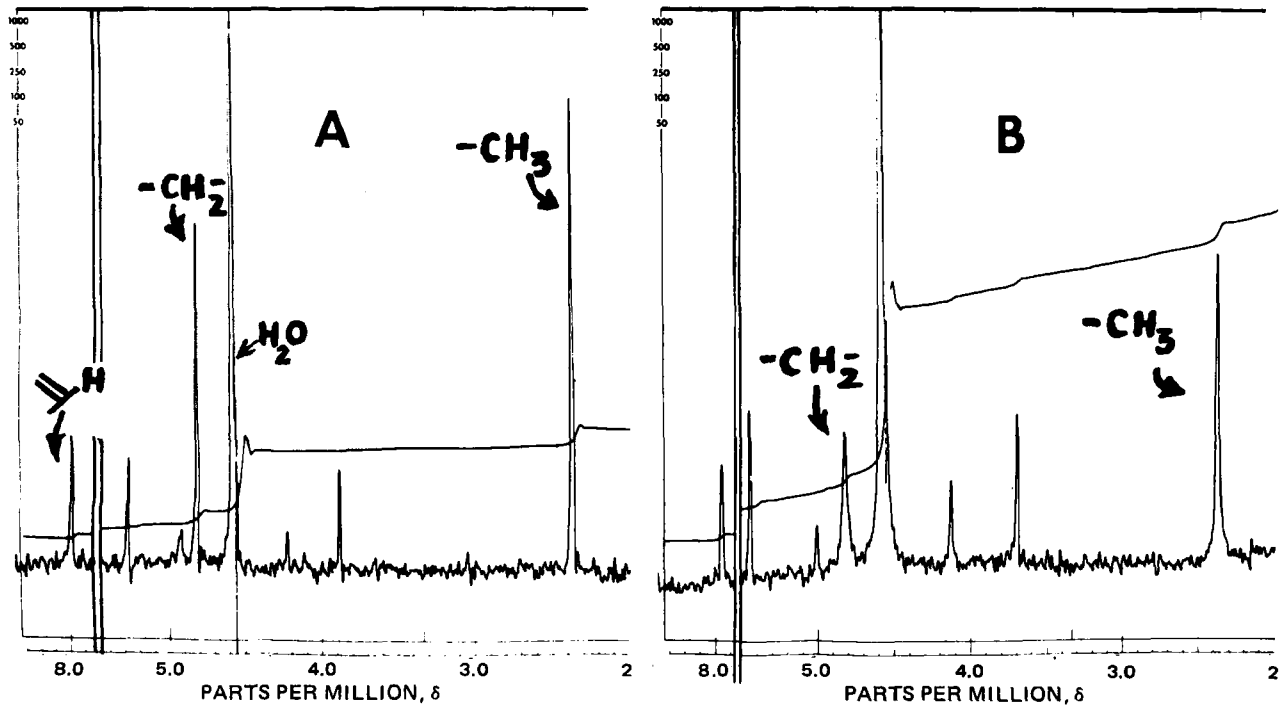
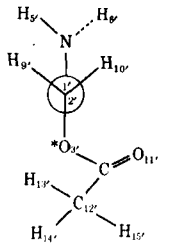


Figure 4—*NMR spectra illustrating the effect of human serum albumin on the alkyl protons of IV at pD 7. Key: A, no protein; and B, with 2.3% human serum albumin.*

ential overlaps are completely neglected. The frontier electron density (q_r) can thus be assembled from the parts of the HOMO (Highest Occupied Molecular Orbital) wave function, which can be expressed as follows:

$$q_r = \sum_i 2C_i^2(\eta) \qquad \text{(Eq. 1)}$$

Table VII—Coordinate System and Minimized Energy of $H_2NCH_2CH_2OCOCH_3$ in Bohr Units[a]



Atom	x	y	z
N_1	0.20424	0.00424	0.00000
$C_{1'}$	2.91006	−0.08213	0.00001
$C_{2'}$	3.69591	2.67055	−0.00000
$*O_{3'}$	6.13957	3.44112	−0.00002
$C_{4'}$	7.32873	5.64244	−0.00000
$H_{5'}$	−1.00271	1.73960	0.00000
$H_{6'}$	−0.97005	−1.73433	0.00000
$H_{7'}$	3.49477	−1.38893	−1.80857
$H_{8'}$	3.49478	−1.38893	1.80857
$H_{9'}$	2.49923	3.55327	−1.83180
$H_{10'}$	2.49927	3.55326	1.83182
$O_{11'}$	6.28668	7.71015	−0.00000
$C_{12'}$	10.15115	5.69633	0.00000
$H_{13'}$	10.93365	7.85597	0.00000
$H_{14'}$	11.27841	4.84339	−1.80702
$H_{15'}$	11.27840	4.84340	1.80703

[a] Total energy = −1.24652 a.u. (MINDO/2). Frontier electron density at the 3′ position = 0.06552 (CNDO).

where C_i (η)'s are HOMO eigenvectors of the CNDO at the ηth position, and Σ_i's constitute the sum of s, p_x, p_y, and p_z atomic orbitals of the atom at the ηth position on the functional group of each compound.

Tables III–VII illustrate the optimized coordinates system and minimized energy computed on the basis of these methods. The possibility of intramolecular interaction, such as hydrogen bonding, was also examined by the CNDO method. Except for IV, which showed a gain of 1.272 kcal/mole (possibly through hydrogen bonding), none of these compounds exhibited any intramolecular interaction.

It is recognized that the binding of drugs to plasma proteins, such as serum albumin, proceeds with the interaction at the primary binding site(s) on the protein, followed by a saturation phase involving any secondary and ternary binding sites available. If it is assumed that the drug–serum albumin interaction occurs only at the primary binding sites at a low drug concentration range, then the plasma protein binding index [$\log_e P_{(mean)}$] (defined as the natural logarithm of the mean percent fraction of the total drug in the bound state) becomes important in determining the availability of the free drug to diffuse into the extravascular compartment. This $\log_e P$ value for each compound at and below 6.4×10^{-5} M concentration was observed to be virtually constant (Table II).

In performing molecular orbital calculations, it was observed that the frontier electron density (q_r) at the 3′-position was the highest for all compounds in this series. Table VIII lists the experi-

Table VIII—Relationship between the Protein Binding Parameter ($\log_e P$) and the Frontier Electron Density (q_r) at the 3′-Position on the Alkyl Side Chain of Metronidazole and Its Derivatives

Compound	Fraction of Drug Bound, %, Mean ± SD	Frontier Electron Density, q_r	Protein Binding Parameter, $\log_e P$	
			Experimental	Calculated
I	3.68 ± 0.58	0.1255	1.30	1.18
II	68.25 ± 0.21	1.2487	4.22	4.18
III	11.07 ± 0.26	0.6374	2.40	2.52
IV	4.08 ± 2.06	0.1554	1.41	1.26
V	2.31 ± 0.21	0.0655	0.84	1.02

mental and calculated $\log_e P_{(mean)}$ values and the frontier electron density at the 3′(*)-position of the N_1-alkyl side chain of metronidazole and its derivatives. The calculated values were determined from Eq. 2. A linear correlation between the $\log_e P_{(mean)}$ and the frontier electron density at the 3′-position (calculated by the CNDO and MINDO/2 methods) was observed for this group of compounds. Such a relationship, as illustrated in Fig. 1, is defined by:

$$\log_e P_{(mean)} = 0.848 + 2.668q_r \qquad \text{(Eq. 2)}$$

$$n = 5 \qquad r = 0.994 \qquad s = 0.131$$

where n is the number of compounds, r is the correlation coefficient, and s is the standard deviation.

A correlation coefficient of 0.994 for the regression line reflects excellent fit for the protein binding data with the computed frontier electron density at the 3′-position of the N_1-alkyl side chain. These results suggest that the plasma protein binding of metronidazole and its derivatives is nonspecific in nature and is dependent upon the frontier electron density at the 3′-position. That is, the drug–protein (mostly serum albumin) interaction occurs through a combination of van der Waals and electrostatic forces.

Evidence that the alkyl side chain and the imidazole moiety do participate in the drug binding process was obtained using NMR spectroscopy. This technique has been used to identify the functional groups on a drug molecule that participate in the drug–protein binding process (25–27). Figures 2–4 illustrate the NMR spectra obtained on solutions of metronidazole and its derivatives with and without human serum albumin at two different concentrations. Due to solubility limitations, these studies could not be performed with III and V (metronidazole acetate).

Whereas the methylene (—CH_2—) and methyl (—CH_3) protons of the metronidazole showed very little change in the presence of serum albumin (Fig. 2), the same protons on II (Fig. 3) showed extensive relaxation proportional to the serum albumin concentration. The magnitude of the relaxation effect observed for II as compared to that observed for metronidazole (I) is consistent with the quantitative results on the extent of binding of these two compounds (Table II). The specific relaxation of the methyl protons at the C-2 position and the methylene protons of the alkyl side chain is indicative of the participation of these functional moieties in the overall binding process.

Similar proton relaxation effect for the alkyl and methyl protons of IV in the presence of plasma proteins is illustrated in Fig. 4. The magnitude of methylene proton relaxation observed for this compound is relatively small compared to that observed for II. Such an observation is consistent with the protein binding data (Table II) and strongly suggests a stabilization effect due to the alkyl side chain and the imidazole moiety.

In conclusion, this study demonstrated that modifications of a functional group on the basic metronidazole molecule caused significant differences in its protein binding characteristics. The degree of binding of this group of nitroimidazoles was shown to be primarily dependent upon the frontier electron density on the terminal portion of the alkyl side chain. The greater the frontier electron density, the greater was the degree of drug binding to plasma proteins. On the basis of these observations, it appears that the frontier electron density parameter with reference to a defined position on a drug molecule can provide useful information concerning the drug–protein interaction of a group of drugs with structural similarities.

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