Journal of Chromatography, 281 (1983) 348–354
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,213

Note

Interaction of 9-aminoacridine, ethidium bromide and harman with DNA characterized by size exclusion high-performance liquid chromatography*

DAVID D. McPHERSON and JOHN M. PEZZUTO*
Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, Health Sciences Center, University of Illinois at Chicago, Chicago, IL 60612 (U.S.A.)
(Received August 2nd, 1983)

A large number of relatively small organic molecules are known to bind to nucleic acids by various mechanisms such as intercalation and ionic attraction[1,2]. It is important to characterize such phenomena and identify additional substances capable of interacting with nucleic acids since biological responses are often associated with this property. For example, the mechanism of action of many mutagenic, carcinogenic and antitumor substances presumably relates to binding with DNA[1,2].

The characteristics of ligand–macromolecule complexes have previously been studied by a variety of techniques[3–5] including classical methods such as equilibrium dialysis[6,7], absorption spectroscopy[3,8] and fluorescence spectroscopy[6,9]. Additionally, as the interaction is best described as a dynamic equilibrium, Hummel and Dreyer[10] introduced a chromatographic method of analysis based on size exclusion in which the eluent contains a fixed quantity of ligand. Prompted by these studies, we surmised that size exclusion resins suitable for use in conjunction with high-performance liquid chromatography (HPLC) systems may prove a useful adjunct for the examination of such interactive phenomena. Thus, we currently report the interaction of 9-aminoacridine, ethidium bromide and harman with DNA, tRNA and various synthetic polyribonucleotides, as determined by size exclusion HPLC.

EXPERIMENTAL

Materials

9-Aminoacridine, ethidium bromide, harman, calf thymus DNA, poly A, poly C, poly U, poly A · poly U, poly I · poly C and tRNA were purchased from Sigma (St. Louis, MO, U.S.A.).

Equipment

Experiments were performed with a HPLC system comprised of a Beckman Model 421 system controller, Beckman Model 100A and 110A pumps, Perkin-Elmer

* This is contribution No. 26 from the Program for Collaborative Research in the Pharmaceutical Sciences.

0021-9673/83/$03.00

Model LC85 variable-wavelength UV spectrophotometric detector and autocontroller, Waters Model 730 data module and Altex Model 210 sample injector fitted with a 20-μl loop. A Cary Model 118 recording spectrophotometer was used for quantitating the samples used in the experiments.

HPLC analysis of the interaction of ligands and nucleic acids

Samples were prepared in standard elution buffer (0.02 *M* Tris–HCl, pH 7.4, 0.10 *M* sodium chloride, 5% dimethylsulfoxide) containing DNA (15 absorbance units/ml), and 9-aminoacridine (45–730 μM), ethidium bromide (40–670 μM) or harman (20–335 μM). For initial experiments in which the binding characteristics of 9-aminoacridine and ethidium bromide were characterized, 20-μl aliquots were injected onto a 10 cm × 7.5 mm O.D. Beckman Spherogel TSK 2000 SW precolumn which had previously been equilibrated with standard elution buffer containing 9-aminoacridine (5–65 μM) or ethidium bromide (2.5–65 μM), respectively (flow-rate, 1.5 ml/min).

Subsequent experiments were performed with a 2.5 cm × 3.9 mm I.D. guard column (Waters Assoc.) which was slurry packed *in vacuo* with 2000SW resin. For the analysis of harman, the eluent contained concentrations ranging from 5–105 μM (flow-rate, 0.5 ml/min). To examine the effect of ionic strength on the binding parameters, the sodium chloride concentration of the standard elution buffer was increased to 0.15 or 0.20 *M*. For additional studies, similar molar nucleotide concentrations of synthetic nucleic acids or tRNA were substituted for DNA.

Absorption was monitored at 260 nm (9-aminoacridine), 283 nm (ethidium bromide) or 245 nm (harman), and all determinations were performed in duplicate.

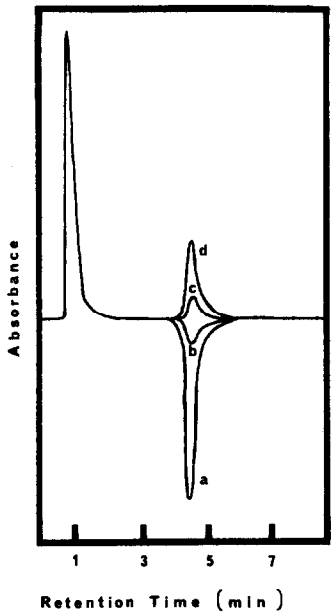
RESULTS AND DISCUSSION

The reversible binding of a ligand to a macromolecule has been theoretically analysed by several authors[11–13], according to the "multiple equilibria theory", which yields the equation

$$\bar{r} = \sum_{i=1}^{m} \frac{n_i K_i[C]}{1 + K_i[C]}$$

where $\bar{r}$ represents the mean number of moles of ligand bound per mole of macromolecule (when dealing with nucleic acids, ligand molecules per nucleotide), m represents the number of classes of independent binding sites such that each class, i, has n_i sites with binding affinity K_i, and $[C]$ is the concentration of unbound ligand. As illustrated by the equation, the binding ratio $\bar{r}$ is independent of the macromolecular concentration. The parameters n_i and K_i are obtained by plotting $\bar{r}/[C]$ *versus* $\bar{r}$ according to Scatchard[12].

One method of determing the quantities $\bar{r}$ and $[C]$ involves equilibration of a column packed with size exclusion resin with a solution containing a fixed amount of ligand[10]. Thus, $[C]$ becomes a controllable variable, *viz.*, the concentration of ligand with which the column is equilibrated. Introduction of a known quantity of a macromolecule capable of interacting with the ligand should then yield a negative



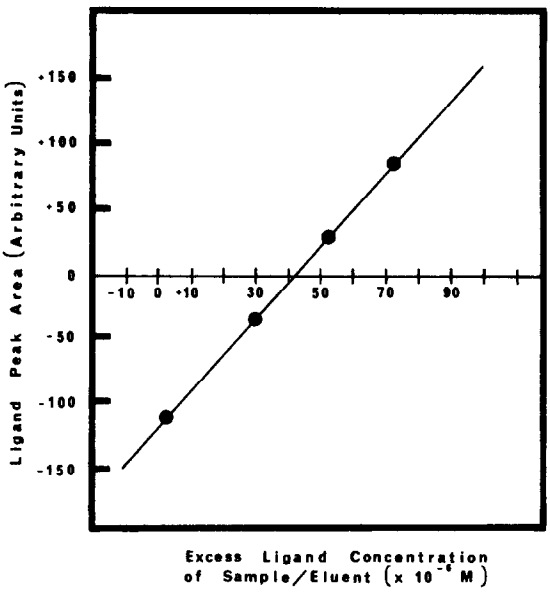


Fig. 1. Hummel and Dreyer-type chromatogram obtained with: (a) 30 μM ethidium bromide; (b) 245 μM ethidium bromide; (c) 370 μM ethidium bromide; (d) 490 μM ethidium bromide injected with a constant amount of DNA (15 absorbance units/ml). Eluent: 25 μM ethidium bromide, 0.20 M sodium chloride.

Fig. 2. Internal calibration of binding of ethidium bromide to DNA. Peak area as a function of the excess (relative to eluent concentration) of ethidium bromide injected with a constant amount of DNA (15 absorbance units/ml). Eluent: 2.5 μM ethidium bromide, 0.20 M sodium chloride.

peak (proportional to the amount of ligand bound) at the elution position characteristic of the ligand.

As shown in Fig. 1, the predicted behavior was exemplified utilizing a HPLC system. The size exclusion column was first equilibrated with 25 μM ethidium bromide. When a small amount of DNA was injected onto the ligand-equilibrated column, a positive peak appeared with a retention time of approximately 1 min which corresponded to the DNA–ligand complex. This was followed by a second (negative) peak with a retention time of approximately 4.5 min which corresponded to the amount of ligand taken from the eluent by the DNA (Fig. 1, line a). As the ligand concentration added to the DNA prior to the injection was increased, the size of the negative peak decreased (Fig. 1, line b). When the initial ligand concentration exceeded the DNA binding capacity, positive peaks were observed at this position (Fig. 1, lines c and d).

An internal calibration is obtained by varying the initial concentration in this manner. The exact amount of ligand bound to the DNA can be determined by plotting the area of the ligand peak (positive or negative) *versus* the excess ligand concentration (sample concentration minus eluent concentration) and interpolating or extrapolating to zero ligand peak area. Typical data obtained from such an analysis are presented in Fig. 2 where it was found that the quantity of ethidium bromide

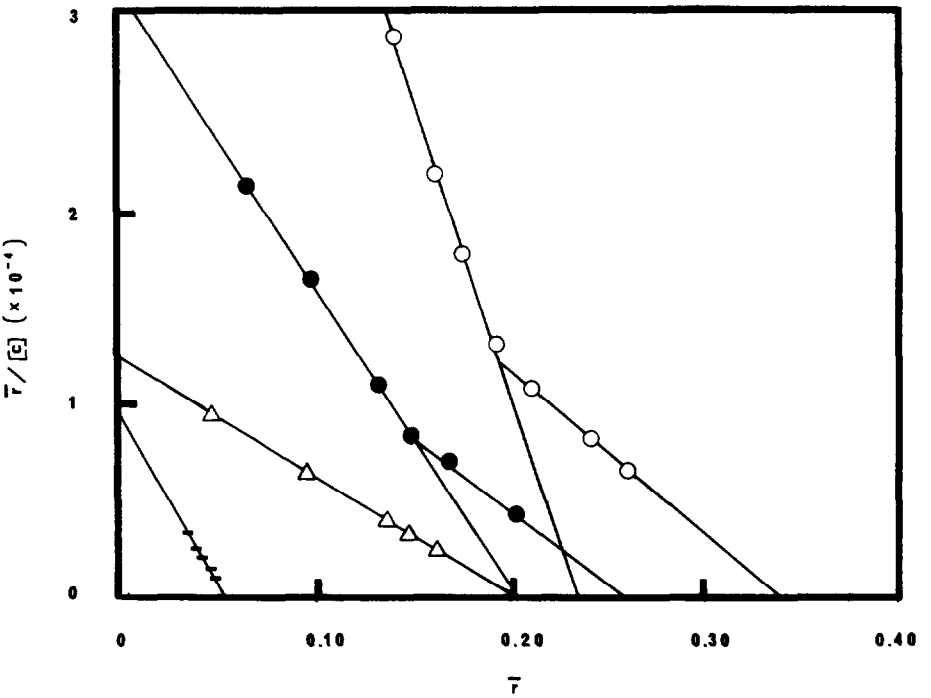


Fig. 3. Scatchard plots illustrating the binding of 9-aminoacridine and ethidium bromide to DNA. (○) Ethidium bromide, 0.10 *M* sodium chloride; (●) ethidium bromide, 0.15 *M* sodium chloride; (■) ethidium bromide, 0.20 *M* sodium chloride; (△) 9-aminoacridine, 0.10 *M* sodium chloride.

bound to DNA corresponded to 42 μM. Since the quantity of DNA is known, the binding ratio, $\bar{r}$, can easily be calculated.

As shown in Fig. 3, application of the technique for the examination of ethidium bromide and 9-aminoacridine interaction with DNA rendered data highly amenable to Scatchard analysis. At a higher salt concentration (0.20 *M* sodium chloride), a single mode of interaction was observed with ethidium bromide, and the binding parameters are summarized in Table I. A similar trend of interaction could be shown with 9-aminoacridine. However, at lower salt concentrations (0.10 and 0.15 *M*), a biphasic response was clearly indicated which is suggestive of multiple modes of interaction. The binding parameters describing these phenomena, which are sum-

TABLE I

BINDING PARAMETERS OF HARMAN, 9-AMINOACRIDINE AND ETHIDIUM BROMIDE TO DNA

Ligand	*NaCl (M)*	K_1	n_1	K_2	$n_1 + n_2$
Harman	0.10	$5.14 \cdot 10^3$	0.116	–	–
9-Aminoacridine	0.10	$6.05 \cdot 10^4$	0.204	–	–
Ethidium bromide	0.10	$2.70 \cdot 10^5$	0.232	$8.50 \cdot 10^4$	0.338
Ethidium bromide	0.15	$1.63 \cdot 10^5$	0.204	$7.50 \cdot 10^4$	0.257
Ethidium bromide	0.20	$1.60 \cdot 10^5$	0.056	–	–

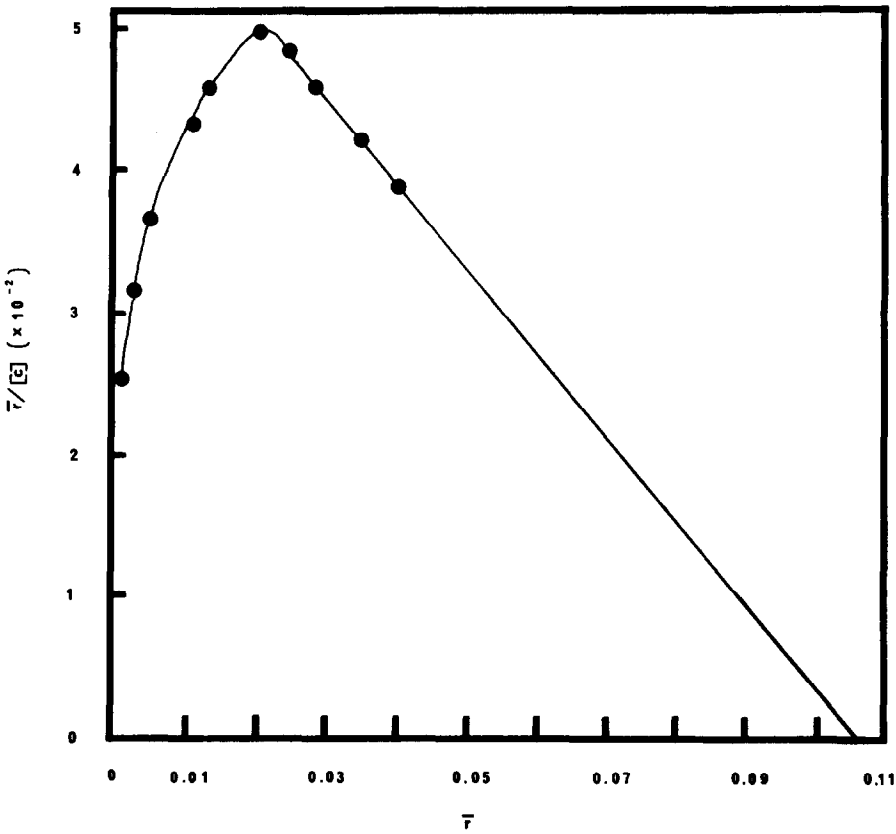


Fig. 4. Scatchard plot illustrating the positive cooperative binding of harman to DNA (sodium chloride, 0.10 *M*).

marized in Table I, are in excellent agreement with those determined by alternative methodology[7,14–16].

Somewhat surprisingly, application of the technique to the analysis of harman resulted in a Scatchard plot which is concave downward and thus indicative of positive cooperativity. The linear segment of the curve which can easily be subjected to kinetic analysis yields binding parameters (Table I) in good agreement with those previously reported[6,17]. Additionally, however, on the basis of studies which have shown enhancement of the expressed mutagenicity of substances such as benzo[*a*]-pyrene and 1-methyl-3-amino-5*H*-pyrido[4,3-*b*] indole[18] upon admixture of this non-mutagenic substance, harman, it is tempting to speculate that the present observation of positive cooperativity relates to its mechanism of comutagenic action. This type of binding modulation will be the subject of future investigations.

In a final series of studies, in addition to DNA, the interaction of 9-aminoacridine, ethidium bromide and harman with tRNA and synthetic polyribonucleotides was investigated semi-quantitatively with a fixed set of elution conditions (Table II). In addition to DNA, substantial binding to the double-stranded species poly A · poly U and tRNA was observed with each of the ligands. Variable (but generally less

TABLE II

BINDING OF VARIOUS LIGANDS TO DNA, tRNA AND SYNTHETIC POLYRIBONUCLEOTIDES

Nucleic acid	*Interacting ligand*		
	*Harman**	*Ethidium bromide**	*9-Aminoacridine**
DNA	0.0190	0.183	0.157
Poly A	0.0080	0	–**
Poly C	0	0	0
Poly U	0	0	0.0060
Poly A · Poly U	0.0135	0.184	0.0560
Poly I · Poly C	0	0.0547	0.0105
tRNA	0.0063	0.0711	0.0218

* Ligand/nucleotide.

** Binding of 9-aminoacridine to Poly A was found to increase with time. After approximately 120 min, the quantity bound was similar to the quantity bound to DNA.

extensive) binding was found with the other nucleic acids that were tested.

In conclusion, utilization of size exclusion HPLC appears to provide a rapid, sensitive probe for studying the interaction of nucleic acids and various ligands under equilibrium conditions. Similar techniques have been applied for quantitating the binding of warfarin and furosemide to protein[19]. In the present study, Scatchard plots obtained with 9-aminoacridine and ethidium bromide are extremely linear attesting to the precision of the method. Further, the interaction (or lack of interaction) of these substances observed with synthetic polynucleotides is consistent with known structural requirements. Moreover, binding ratios of as low as 0.0013 were accurately determined with harman, which permitted characterization of a positive cooperative interaction which heretofore had not been reported utilizing other techniques. Finally the detection method for quantitating the binding is not limited to UV absorption, and ultimately this procedure may prove useful for tasks such as determining the presence of DNA binding species in crude mixtures, characterization of the interactive binding of two or more ligands and examining regiospecific interactions of ligands with DNA.

ACKNOWLEDGEMENTS

The authors are grateful to Ms. Dorothy Guilty for help in the preparation of this manuscript. One of us (D.D.M.) was the recipient of a Predoctoral Fellowship awarded by the National Institutes of Health, 1982–1983.

REFERENCES

1 H. M. Berman and P. R. Young, *Annu. Rev. Biophys. Bioeng.*, 10 (1981) 87.
2 C. C. Irving, in H. Busch (Editor), *Methods in Cancer Research*, Academic Press, New York, 1973, p. 189.
3 E. J. Gabbay, R. E. Scofield and C. S. Baxter, *J. Amer. Chem. Soc.*, 95 (1973) 7850.

4 A. Blake and A. R. Peacocke, *Biopolymers*, 6 (1968) 1225.
5 B. C. Baguley, W. A. Denny, G. J. Atwell and B. F. Cain, *J. Med. Chem.*, 24 (1981) 170.
6 J. M. Pezzuto, P. P. Lau, Y. Luh, P. D. Moore, G. N. Wogan and S. M. Hecht, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 1427.
7 W. R. Wilson, B. C. Baguley, L. P. G. Wakelin and M. J. Waring, *Mol. Pharmacol.*, 20 (1981) 404.
8 J. M. Pezzuto, S. K. Antosiak, W. M. Messner, M. B. Slaytor and G. R. Honig, *Chem. Biol. Interact.*, 43 (1983) 323.
9 B. C. Baguley and E. M. Falkenhaug, *Nucleic Acid Res.*, 5 (1978) 161.
10 J. P. Hummel and W. J. Dreyer, *Biochim. Biophys. Acta*, 63 (1962) 530.
11 I. M. Klotz, *Arch. Biochem.*, 9 (1946) 109.
12 G. Scatchard, *Ann. N.Y. Acad. Sci.*, 51 (1949) 660.
13 R. L. Scott, *Rec. Trav. Chim. Pays-Bas*, 75 (1956) 787.
14 M. J. Waring, *J. Mol. Biol.*, 13 (1965) 269.
15 L. M. Angerer and E. N. Moudrianakis, *J. Mol. Biol.*, 63 (1972) 505.
16 L. M. Angerer, S. Georghiou and E. N. Moudrianakis, *Biochemistry*, 13 (1974) 1075.
17 K. Hayashi, M. Nagao and T. Sugimura, *Nucleic Acid Res.*, 4 (1977) 3679.
18 M. Nagao, T. Yahagi, T. Kawachi, T. Sugimura, T. Kosuge, K. Tsuji, K. Wakabayaski, S. Mizusuki and T. Matsumoto, *Proc. Jpn. Acad.*, 53 (1977) 95.
19 B. Sebille, N. Thuaud and J.-P. Tillement, *J. Chromatogr.*, 167 (1978) 159.