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DNA AS AN AFFINITY PROBE USEFUL IN THE DETECTION AND ISOLATION OF BIOLOGICALLY ACTIVE NATURAL PRODUCTS

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ABSTRACT.—A simplified hplc system is described for the detection of compounds capable of binding to DNA. Compounds known to interact with DNA were found to invoke a positive response with this system, and the concentration-dependence and sensitivity were determined. When applied to 17 randomly selected plant extracts, five elicited a positive response and, of these, four were subsequently found to be cytotoxic with cultured KB or P-388 cells. As described in a companion paper, one of these extracts (derived from *Albizia amara*) has been further processed and found to contain a group of structurally-unique macrocyclic alkaloids that demonstrate a variety of biological activities. Therefore, this approach should prove of value in facilitating the identification of plant extracts that contain substances capable of binding to DNA and the subsequent activity-directed fractionation for the procurement of active principles. As a prescreen or monitor, these relatively uncomplicated hplc procedures can be used in laboratories not prepared to perform more complicated or costly bioassay techniques. Thus, the pool of potentially active novel chemical substances to be considered for more advanced testing could be increased.

The mechanism of several known antitumor and antimalarial agents involves interaction with DNA. Examples include alkylating agents (e.g., chlorambucil, cyclophosphamide, melphalan, streptozocin), antitumor antibiotics (e.g., bleomycin, doxorubicin, mithramycin), and various other substances (e.g., cisplatin, chloroquine). Based on the interaction of small molecular weight ligands with DNA, some short-term procedures have been devised that are applicable for the discovery of naturally occurring agents that function by this mechanism (1–4). In addition, a variety of methods have been utilized for analyzing the interaction of small molecular weight compounds with DNA, such as equilibrium dialysis (5) and spectrophotometric titration (6). Employing size-exclusion hplc and the gravity-fed elution technique originally described by Hummel and Dreyer (7), we have devised a method for studying ligand-macromolecule (nucleic acid) interactions (8).

In brief, a fixed amount of ligand is added to the elution solvent of the hplc system, and a known quantity of DNA is then injected. This produces a negative peak at the elution position characteristic of the ligand; the area of the peak is proportional to the amount of bound ligand. As exemplified with 9-aminoacridine or ethidium bromide, the resulting data are highly amenable to Scatchard analysis, and binding to nucleic acids other than DNA (e.g., tRNA, synthetic polynucleotides) can be analyzed. Further, binding ratios as low as 0.0013 were accurately determined with harman, and thorough analysis of the interaction of this compound with DNA revealed positive cooperativity. Importantly, because harman has previously been reported to demon-

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strate comutagenic activity (9), this type of binding activity may be of biological relevance.

It was surmised that this approach could be extended to screen plant extracts for the presence of DNA binding substances. In turn, utilization of the procedure could lead to the rapid isolation of active principles. Unexpectedly, due to non-specific binding phenomena, size-exclusion hplc was found to be unsuitable for work involving crude extracts. However, as previously reported in preliminary form,[4] DNA elutes as an unretained peak when applied to an analytical C_{18} hplc column, and co-injection with substances known to bind to DNA (or pre-injection of DNA-binding species that are retained by the adsorbant) diminishes the size of the DNA peak. We now report the details of these procedures which have been investigated with a number of known and previously uncharacterized substances. Experimental conditions have been established that are suitable for drug discovery programs. As illustrated in a companion paper (10), application of these procedures has led to the discovery of a group of structurally unique macrocyclic alkaloids that interact with DNA and demonstrate a variety of biological activities.

EXPERIMENTAL

CHEMICALS AND HPLC APPARATUS.—Calf thymus DNA and unfractionated yeast tRNA were purchased from Sigma Chemical Co. (St. Louis, MO), and hplc-grade solvents were obtained from Fisher Scientific (Itasca, IL). Test compounds were supplied either by investigators affiliated with the Program for Collaborative Research in the Pharmaceutical Sciences, University of Illinois at Chicago, or the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. The primary hplc instrument used was a Waters Model 600 Multisolvent Delivery System equipped with a U6K injector, a Model 441 absorbance detector (set at 254 nm unless otherwise specified), and a Model 740 Recorder/Data Module. Typically, a C_{18} reversed-phase column (Altex Ultrasphere-ODS, 5μ particle size, 4.6 × 250 mm) equipped with a Waters Guard-Pak precolumn was used for these studies.

HPLC ANALYSIS PROCEDURES.—DNA or tRNA solutions were prepared in H_2O (0.1 mg/ml), and test substances were dissolved in either H_2O or MeOH (1.0 mg/ml, unless otherwise specified). The hplc column was equilibrated with an H_2O-MeOH (80:20) solution. Test samples and DNA (tRNA) solutions were then applied to the hplc column by one of the following procedures: (a) pre-mixing the DNA (tRNA) solution with the test sample solution (1:1, v/v), incubating the mixture at ambient temperature (5–30 min), and injecting the mixture (40 or 80 μl) (Pre-incubation Method), (b) co-injecting equal volumes of the DNA (tRNA) and test sample solutions into the U6K sample loop (20 or 40 μl each), following by injection of the mixture (Co-injection Method), or (c) injection of the sample solution (20 or 40 μl) followed by injection of the DNA (tRNA) solution after a delay of 2–4 min (Delayed Injection Method). In general, the flow rate was maintained at 1 ml/min. Under these conditions, free DNA (tRNA) eluted from the column in approximately 1.2 min. One min after the appearance of the DNA peak, the MeOH concentration in the eluent was increased to 100% (gradient duration 1 min). Constituents of the sample mixture were then eluted from the column during a period of 20 min. In all experiments, the DNA (tRNA) peak size was compared with that obtained from an analogous elution procedure performed with DNA (tRNA) alone.

ANALYSIS OF DNA/COMPOUND ELUTION CHARACTERISTICS USING RP-18 TLC PLATES.—Tlc plates (RP-18 F_{254s}; 0.25 mm; Merck, Art 15389) were pre-developed with MeOH-H_2O (8:2). Test substance was then applied (5 mg/ml; ca 1 μl) at the origin (3 spots), followed by the addition of DNA (1 mg/ml; ca. 5 μl) at the same positions at the origin. The plate was then developed with the same solvent system, and the position of the DNA was determined by spraying the plates with anisaldehyde reagent. As established in preliminary studies, this reagent yields a blue color reaction with DNA, and the intensity of the color was proportional to the quantity of DNA added to the plate.

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[4] J.M. Pezzuto, C.-T. Che, D.D. McPherson, G. Topcu, C.A.J. Erdelmeier, and G.A. Cordell, NIH Workshop: Bioassays for Discovery of Antitumor and Antiviral Agents from Natural Sources, Lister Hill Auditorium, National Library of Medicine, Bethesda, MD, Oct. 18-19, 1988; and J.M. Pezzuto, C.-T. Che, D.D. McPherson, G. Topcu, C.A.J. Erdelmeier, and G.A. Cordell, Workshop on "Simple Bioassays," International Research Congress on Natural Products, San Juan, Puerto Rico, 6-10 August 1989.

ANALYSIS OF CYTOTOXIC POTENTIAL.—In order to characterize the hplc test procedure, a number of plant extracts randomly selected from our repository were analyzed. These extracts were also evaluated for cytotoxic potential with cultured P-388 or KB cells using previously published procedures (6, 11).

RESULTS AND DISCUSSION

In order to investigate the characteristics of this hplc binding-analysis system, 26 pure compounds were evaluated. As summarized in Table 1, many of these substances were selected on the basis of known interactions with nucleic acids. For example, doxorubicin is known to intercalate with DNA (12), and addition of doxorubicin to DNA completely eliminated the DNA peak (Figure 1, left panel). A strongly positive response was also observed with several other test substances known to interact strongly with DNA (e.g., 9-aminoacridine, daunomycin, ellipticine, ethidium bromide, fagaronine), and a good correlation was obtained between the response observed with DNA or tRNA. For routine screening procedures, there are some advantages to using tRNA, since this nucleic acid is technically easier to handle in comparison with high-molecular weight DNA (e.g., it is more readily soluble in H_2O and the solutions produced are less viscous).

In addition, four sesquiterpenes were examined and found to display a weak positive response (Table 1). This may be related to an interaction between the exocyclic methylene moiety of these compounds and nucleophilic sites of DNA, since com-

TABLE 1. Effect of Test Substances on the DNA or tRNA Peak Size.[a]

Compound tested	DNA Peak Reduction (%)	tRNA Peak Reduction (%)
Acridine, 9-amino[b]	>70	100
Acronycine	<30	ND[c]
Actinomycin D[b]	30-70	<30
Bleomycin[b]	<30	>70
Camptothecin[b]	no effect	no effect
Canthin-6-one, 10-methoxy	30-70	ND
Chaparrinone, 6α-senecioyloxy	no effect	no effect
Colchicine	<30	<30
Daunomycin[b]	100	ND
Doxorubicin[b]	100	100
Ellipticine[b]	>70	30-70
Ethidium bromide[b]	100	100
Fagaronine[b]	100	100
Jacaranone	30-70	<30
Kauren-19-oic acid	no effect	ND
Kaur-16-en-19-oic acid, 15-cinnamoyl	no effect	ND
Monoginoic acid	no effect	ND
Montacephalin	30-70	no effect
Parthenolide	30-70	<30
Podophyllotoxin	no effect	no effect
Steviol	no effect	ND
Strebloside	no effect	no effect
Tomencephalin	30-70	ND
Tomencephalin, 5-hydroxy	30-70	<30
Vinblastine	>70	100
Vincristine	>70	100

[a]The indicated compounds were analyzed by the "Pre-incubation Method" of hplc analysis described in "Experimental."

[b]Compound known to interact with DNA.

[c]ND not determined.

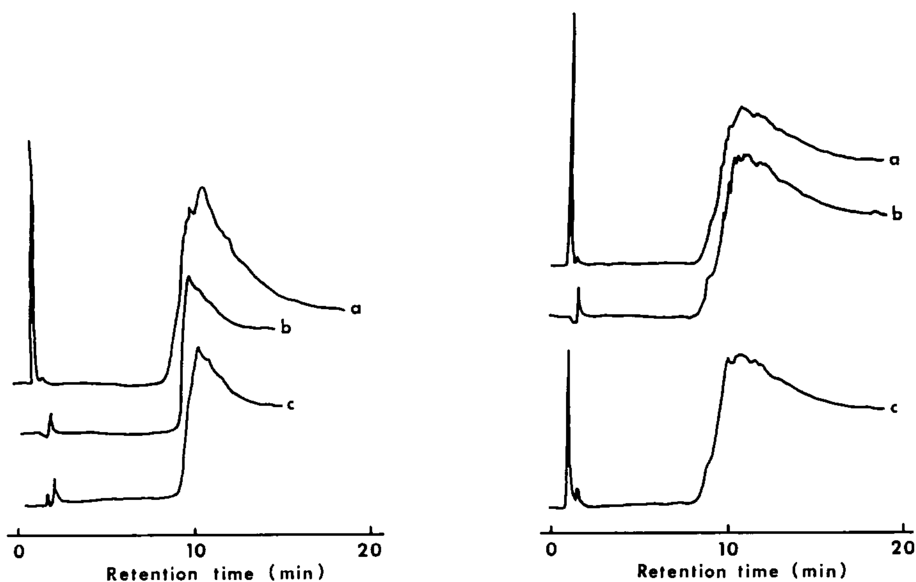

FIGURE 1. Left Panel. Illustration of typical hplc profiles obtained by elution of (a) DNA, (b) doxorubicin, or (c) a mixture of DNA and doxorubicin. Right Panel. Illustration of hplc profiles obtained by elution of (a) DNA, (b) bleomycin, or (c) a mixture of DNA and bleomycin. Relative to profile a, the area of the first peak in profile c is reduced by approximately 30%. Additional details are provided in Experimental.

pounds of this class are known to interact with other nucleophiles (13). When bleomycin (Figure 1, right panel) and camptothecin were evaluated in this assay system, either no effect or a minimal response was observed with DNA. Antitumor activities of these compounds are believed to be due to the cleavage of covalent bonds in polydeoxyribonucleotides, although a binding mechanism may also be involved prior to bond cleavage (14, 15). Podophyllotoxin and colchicine, both known to interact with tubulin rather than DNA (16, 17), exhibited very little effect, as did the kaurane diterpenes and strebloside (a cardenolide).

Overall, these observations confirm the relationship between the empirical hplc elution characteristics demonstrated in this system and the potential of the test substances to interact with DNA (tRNA). Notable exceptions, however, are the responses that were observed with vinblastine (VLB) and vincristine (VCR). It is well established that these substances interact with tubulin (16–18), causing metaphase arrest (19). In the present study, both compounds were found to reduce the DNA and tRNA peak sizes (Table 1). This was not anticipated since, as suggested above, such a response should be indicative of interaction. In order to investigate these phenomena, we have performed some preliminary studies to establish whether the response was a false positive or if these substances actually interact with nucleic acids. First, differential spectral titrations were performed, and it was found that the uv absorption spectra of VLB or VCR were reduced in a dose-dependent manner on addition of calf thymus DNA (data not shown). This type of response is indicative of interaction. Next, VCR was applied to a DNA-cellulose column as described by Mar *et al.* (10), and total retention was observed until the elution was changed to 100% MeOH (data not shown). This behavior is also indicative of interaction with nucleic acids. Thus, the positive response observed with the hplc system may indeed be due to interaction with the nucleic acids. Consistent with this suggestion, VLB and VCR are known to inhibit DNA and RNA synthe-

sis, and it has been suggested that this activity (in conjunction with antimitotic activity) contributes to the favorable therapeutic potential of these substances (20).

As is the case with any assay system that is reflective of a biological response, it is important to establish if the response of the test system is dependent on the dose of the test substance. In the presence of increasing quantities of DNA intercalators, a greater portion of DNA is bound to form a complex, and consequently, the size of the free DNA peak should be reduced until it is no longer detectable. Thus, ethidium bromide and ellipticine were used to assess dose-response relationships. As illustrated in Figure 2, increasing concentrations of either test substance clearly diminished the size of the DNA peak in a dose-dependent manner. On the basis of these data, it may be concluded that (a) observations of peak size accurately reflect interactions with DNA, and (b) this assay is capable of providing semi-quantitative estimations of DNA interaction.

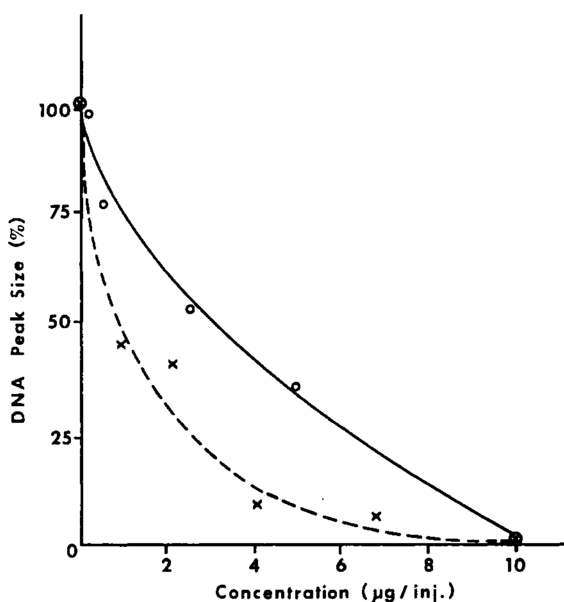

FIGURE 2. Dose-dependent diminution of DNA peak size mediated by co-injection of the indicated quantities of ellipticine (×) or ethidium bromide (○). Additional details are provided in Experimental.

The behavior of the detection system was then investigated with plant extracts. First, in order to assure the detection of known DNA binding agents in complex mixtures, two plant extracts known to be inactive were enriched with DNA-binding agents and assayed. In one test case, a $CHCl_3$ extract of *Actinostemon concolor* (inactive when tested for cytotoxicity with cultured P-388 and KB cells) was subjected to preliminary separation on a Si gel column by sequential elution with $CHCl_3$ and MeOH. The MeOH eluate (inactive in the present hplc assay system) was concentrated, redissolved in MeOH (10 mg/ml), and mixed with an aqueous solution of doxorubicin to yield a final concentration of 0.001% (w/w) doxorubicin. This test substance showed a 100% reduction of the DNA peak when co-injected with DNA. In a second test case, a $CHCl_3$ extract of *Euphorbia cotinifolia* was fractionated on a Si gel column to afford several fractions, one of which was found to be non-cytotoxic and inactive in the present hplc detection system. The inactive fraction was mixed with a solution of ellipticine, yielding a

final concentration of 0.005% (w/w) ellipticine in the plant fraction. When assayed in the present hplc system, the mixture induced a strong reduction (ca. 80%) in the DNA peak. Utilizing the same *Euphorbia* extract, enrichment experiments were also performed with fagaronine. When admixed at a concentration of 0.05% (w/w), the DNA peak was not detectable. When the fagaronine concentration was reduced to 0.005% (w/w) and the mixture was tested for activity, the size of the DNA peak was reduced by more than 70%. Similarly, an actual MeOH extract prepared from *Fagara zanthoxyloides* (known to contain an unknown quantity of fagaronine) (6) induced a strongly positive response with the test system. Thus, on the basis of these results, the hplc method has a detection limit in the range of 0.005–0.001% (w/w), which is considered reasonable for the evaluation of active principles in plant products.

Seventeen randomly selected plant extracts were then evaluated by this procedure. As summarized in Table 2, five plant extracts elicited a positive response with the hplc detection system. Of these, four were subsequently found to be cytotoxic with cultured KB or P-388 cells; three others we found to demonstrate a significant cytotoxic response without demonstrating a positive response in the hplc detection system. This type of activity distribution seems reasonable for the identification of plants containing the desired active principles, but final judgement awaits more complete phytochemical analysis of a number of plants demonstrating activity in the hplc detection system. We have thus far proceeded to analyze the first plant listed in Table 1 that demonstrated a cytotoxic response and a positive response in the hplc detection system (*Albizia amara*). As a result, a group of structurally unique macrocyclic alkaloids have been discovered that demonstrate an interesting array of biological activities (10).

It is apparent that the hplc detection system described in this report is subject to additional study and refinement. For example, it is currently hypothesized that the diminution of the DNA (tRNA) peak is due to interaction of active substances with DNA (tRNA) and the reversed-phase resin, and this results in retention. Some preliminary studies have been conducted that tend to confirm this hypothesis. As illustrated in Figure 3, when DNA was applied to an RP-18 tlc plate, migration was observed when

TABLE 2. Evaluation of Plant Extracts for Cytotoxic Potential Ability to Reduce DNA Peak Size.

Plant Tested	Plant Part	Extract	ED$_{50}$ (μg/ml)		DNA Peak Reduction
			KB	P-388	
Albizia amara	seed	MeOH	12.6	1.8	yes
Caraipa fasciculata	twig	MeOH	>100	>100	no
Cerbera manghas	stembark	MeOH	>100	>100	no
Cunaria sprucena	root	MeOH	>100	1.5	no
Erythrina rubrinervia	twig	MeOH	>100	27.5	no
Euphorbia cotinifolia	above-ground	$CHCl_3$	>100	11.9	yes
Funtumia africana	stembark	MeOH	100	8.8	yes
Jamesonia imbricata	whole	$CHCl_3$	66	>100	no
Laurelia nova-zealandiae	stembark	MeOH	>100	31	no
Olea europaea	twig-leaf	MeOH	>100	31	no
Pourouma palmata	root	$CHCl_3$	81	68	no
Salix humboldtiana	twig-leaf	MeOH	>100	57	no
Salix humboldtiana	leaf-flower	MeOH	8.4	10	yes
Schefflera bogotensis	stembark	MeOH	>100	>100	no
Strophanthus hispidus	twig-leaf	MeOH	8.3	49	no
Typha domingensis	root	MeOH	>100	50	yes
Xylopia aromatica	twig-leaf-flower	$CHCl_3$	27	3.3	no

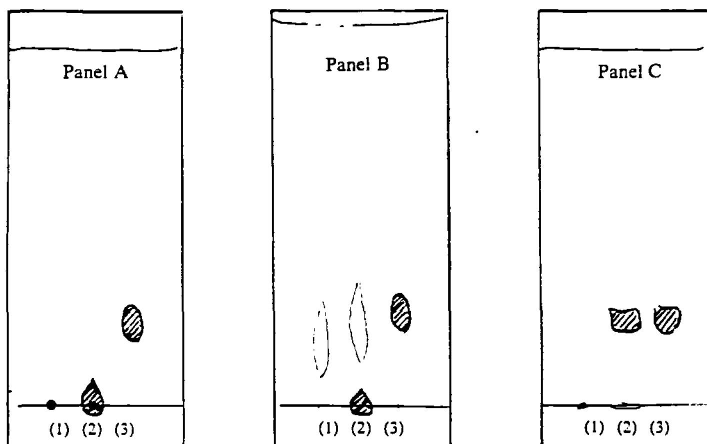

FIGURE 3. RP-18 tlc profiles observed with DNA and test compounds. Panel A: (1) fagaronine, (2) fagaronine + DNA, (3) DNA. Panel B: (1) ethidium bromide, (2) ethidium bromide + DNA, (3) DNA. Panel C: (1) steviol, (2) steviol + DNA, (3) DNA. DNA was detected by spraying the plates with anisaldehye reagent. Additional details are provided in Experimental.

MeOH-H_2O (8:2) was used as the elution solvent. However, when the DNA was mixed with compounds with which it is known to interact (either fagaronine or ethidium bromide), the complex was retained at the origin when MeOH-H_2O (8:2) was used for elution. Steviol, a compound that is not active in the hplc procedure, did not cause the DNA to be retained at the origin.

We have further investigated the mode of sample application. Three methods have been employed: pre-incubation, co-injection, and delayed injection (see Experimental). The pre-incubation method has the advantage of prolonging the DNA-ligand interaction period and thus will potentially allow a higher degree of association. Elevation of the incubation temperature is also convenient with this procedure, and this may also enhance the association. We have found that fixed concentrations of certain compounds (including doxorubicin and daunomycin) totally abolished the DNA peak when tested by the pre-incubation procedure, but when tested by the co-injection procedure, the same concentrations permitted the observation of residual DNA peaks. Therefore, the pre-incubation method enhances sensitivity. However, during the course of this investigation, precipitation was occasionally noted after the incubation period, probably due to the low solubility of certain test substances in aqueous solution. Under these circumstances, the pre-incubation method is of limited usefulness.

The second method of sample application, co-injection, is basically a zero-time incubation. DNA initially comes into contact with the sample in the injector loop, and both substances are immediately applied to the column. This was the procedure most commonly employed during this investigation. When dealing with certain classes of test substances (e.g., hydrophobic compounds capable of intercalating with DNA) excellent results were obtained. A problem with this procedure (and the pre-incubation procedure) was encountered when the test material contained polar substances that co-eluted with DNA. If these polar materials demonstrated absorbance at the same wavelength used to monitor the DNA peak, it was difficult to estimate accurately the size of the peak of interest. A number of methods can be developed to circumvent this problem (e.g., use of DNA with a modified retention time, use of radiolabelled DNA for detection, etc.), but we have found that preliminary clean-up procedures for plant

extracts (e.g., solvent extraction, passage through charcoal or cartridges containing reversed-phase resin) are of immediate advantage. It is also likely that tannins (polyphenolics) would interfere with these procedures, and a number of pre-treatment procedures for removal have recently been described that are equally applicable for the plant extracts to be evaluated by these hplc procedures (21).

The third method of application, delayed injection, also effectively accomplishes the removal of rapidly eluting (polar) substances. By this method, the sample is permitted to be adsorbed by the resin (perhaps with some degree of separation) for a relatively brief period of time (e.g., 3–4 min) prior to the introduction of DNA. Thus, unretained polar components are eluted from the column prior to the time of observing the DNA peak, and the complication of discerning the presence of the DNA peak due to a masking effect by polar substances is eliminated, since its appearance can be delayed until a stable baseline is achieved. This procedure is very effective with known agents that strongly interact with DNA. When dealing with unknown test substances, however, this method would limit detection to active principles retained by the resin. Since the initial elution solvent we have employed is H_2O-MeOH (80:20), it is likely that many natural products of interest would be retained by the resin under these conditions, so that concern may not be of major consequence. However, another factor that could prohibit the detection of a response with the delayed injection technique would be the limited capacity for nucleic acid-ligand interaction under these conditions. One straightforward method worth attempting to increase the potential for interaction would be to reduce the flow rate.

As indicated by the nature of the preceding discussion, it is not possible at the present time to propose the best general procedure for screening plant extracts using this hplc-based assay. The most applicable procedure will largely depend on the nature of the components to be evaluated, and some preliminary experiments are likely to be required. Nonetheless, even in unrefined form, the approach described herein should prove of value in facilitating the identification of plant extracts that contain substances capable of binding to DNA and the assay of subsequent fractions that are generated for the procurement of active principles. Alternatively, plants that are identified as active by these procedures may be used for the procurement of marker quantities of ligands (e.g., utilizing DNA-cellulose cc), and these markers can be employed to direct the isolation of larger quantities of active principles. This approach totally eliminates the need for bioassay during fractionation procedures (cf. 10), thus saving on both the biological tests and the time to obtain the results of said tests. Although the antitumor activity of substances that interact with DNA cannot be predicted a priori, the need for DNA site-specific agents is apparent, and the potential of such agents to affect selectively the expression of cellular or viral oncogenes is worthy of consideration. In addition, substances isolated on the basis of DNA interaction may demonstrate a large number of other biological activities (10). As a prescreen or monitor, relatively uncomplicated hplc procedures can be used in laboratories not prepared to perform more complicated or costly bioassay techniques, and additional in vitro procedures can be applied to characterize the interaction of the isolates with nucleic acids. Thus, the pool of potentially active novel chemical substances to be considered for more advanced testing should be increased.

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