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NEW SELECTIVE DETECTION PRINCIPLE FOR LIQUID CHROMATOGRAPHY

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SUMMARY

A new detection principle for liquid chromatography based on the continuous measurement of the fluorescence emission intensity of a fluorescent dye, and its enhancement in the presence of solute, is described. The selective detection of polar lipids using 1-anilinonaphthalene-8-sulfonic acid as the fluorescent dye was demonstrated. Detector sensitivity, linearity, and several response-affecting factors were studied for this type of detection.

INTRODUCTION

The lack of sensitive detection in modern liquid chromatography (LC) is a frequent hindrance to many potential applications of this powerful analytical method. Whereas the development of a universal, highly sensitive detector for LC would be the most welcomed solution to the sensitivity problems, many useful applications can be covered by one of the available high-sensitivity selective detectors, such as small-volume spectrophotometric, spectrofluorometric, or electrochemical flow cells. In addition, many classes of compounds that do not possess selectively detectable features in their molecules can be derivatized to introduce them.

Among the available detection principles for LC, fluorescence surpasses most, if not all, other measurement methods in terms of sensitivity. Unfortunately, only the compounds which fluoresce or those easily convertible to fluorescent derivatives can be detected. Perhaps the best example of the potential of highly sensitive detection in LC is the detection of fluorescent derivatives[1,2].

It has been observed in numerous instances that the fluorescence of certain compounds can be influenced by a physical interaction with other (non-fluorescent) solution components. These can either quench or enhance the fluorescence intensity. Whereas such phenomena may actually contribute to errors in analytical measure-

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ments in mixtures, they could be potentially utilized, under controlled conditions, to one's advantage. The present publication describes some preliminary results obtained on the application of such phenomena to improved detection in LC.

Lipids are just one of the classes of compounds of biologic interest that have been limited in terms of sensitive detection[3]. Unless derivatizations are carried out[4–6] that are presently feasible in a few cases, only the refractive index monitor or transport-type detectors[3,7] are feasible for continuous detection. Therefore, our primary interest in exploring the above-mentioned detection principle has been focussed on chromatography of lipids.

Diverse chemicals have been used as fluorescent probes to provide information on the microenvironments of biopolymers and micellar systems as well as for labelling of membranes[8–11]. Among them, 1-anilinonaphthalene-8-sulfonic acid (ANS) appears most popular. Also, hydrophobic bonding with other molecules is responsible for the increased fluorescence of dansyl (a term used for 1-dimethylaminonaphthalene-5-sulfonyl) amino acids in organic solvents[12].

Whereas systematic searches leading to the utilization of fluorescence enhancement and quenching phenomena in LC detection are underway in this laboratory, this communication pertains to the utilization of ANS (as an example of fluorescent dye) in connection with improved detection of certain polar lipids. Firstly, the magnitude and nature of the fluorescence enhancement phenomena were investigated by means of a scanning spectrofluorometer. Subsequently, selected conditions were applied to monitoring the output of an LC column used for separating polar lipids. Sensitivity, selectivity, and the linearity of response were studied, and several response-affecting variables were also briefly investigated.

EXPERIMENTAL AND RESULTS

Spectrofluorometric measurements

In order to estimate detectability of selected lipids in an LC fluorometric detector and find optimum detection conditions, fluorescence spectra of ANS were recorded under a variety of conditions of pH and solvent composition in the absence and presence of small amounts of lipid. All measurements were carried out with a Farrand Optical Co. Model 104243 single-beam scanning spectrofluorometer. The light source was a high-pressure xenon/mercury arc lamp. The spectra obtained were not corrected for spectral characteristics of the source or detector.

The fluorescence enhancement effect of lecithin on ANS is shown in Figs. 1 and 2. Fig. 1 is the excitation spectrum of ANS (5×10^{-4} *M* concentration) in a 0.1 *F* phosphate buffer (pH 6.8), with enhancement obtained by the addition of egg-yolk lecithin (50 μg/ml). Excitation occurs between 300 and 450 nm, with enhancement seen as a general increase in intensity at all wavelengths. The region around the 405 nm mercury line exhibits the strongest enhancement.

Fig. 2 shows the characteristically broad emission spectrum of ANS. Whereas unenhanced emission is centered around 520 nm, enhancement by phospholipid induces a shift in the emission maximum to shorter wavelengths. The degree of shift appears to be somewhat a function of the lipid concentration. The choice of excitation wavelength used does not qualitatively affect the emission spectrum, permitting selection of an excitation wavelength based primarily on maximizing the enhancement sig-

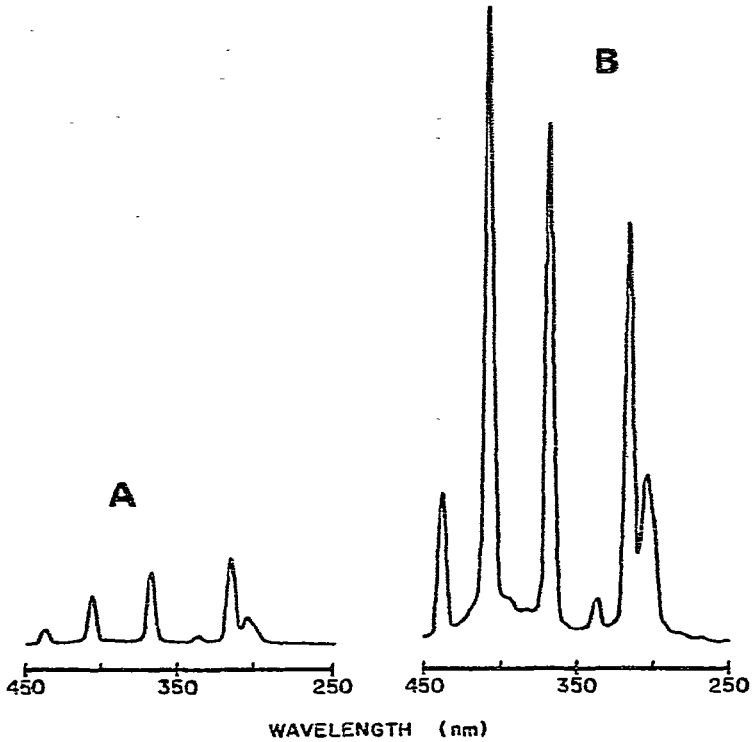

Fig. 1. Excitation spectra of ANS (5×10^{-4} *M*) in 0.1 *F* phosphate buffer (pH 6.8) in the absence of lecithin (A), and with lecithin (50 μg/ml) added (B). Emission monitored at 485 nm.

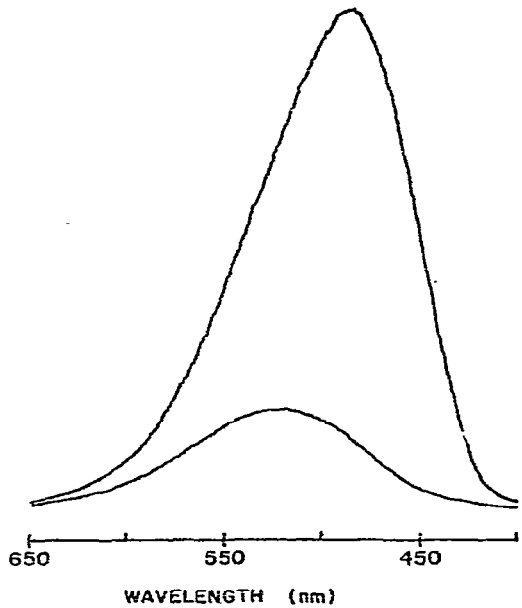

Fig. 2. Emission spectra of ANS (5×10^{-4} *M*) in 0.1 *F* phosphate buffer (pH 6.8) in the absence of lecithin (lower curve), and with lecithin (50 μg/ml) added (upper curve). The excitation wavelength used was 365 nm.

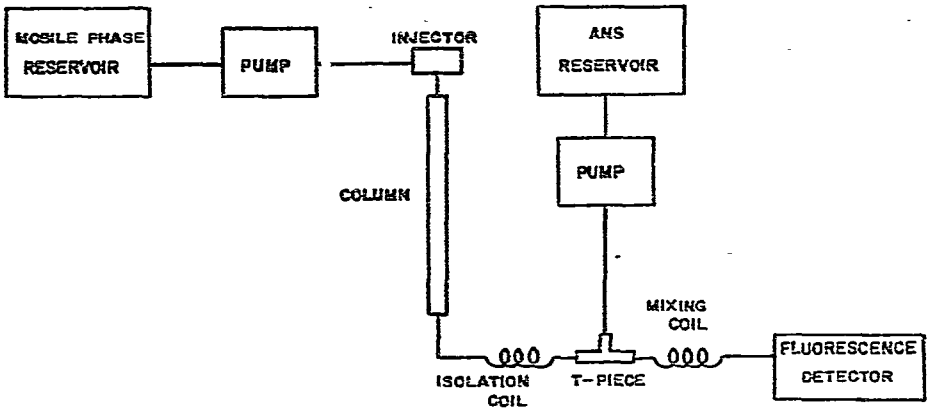

Fig. 3. Schematic diagram of chromatographic system.

nal. With a mercury lamp source, the 365 or 405 nm excitation lines should result in the most sensitive detection.

Buffer pH was found to have only a minor influence on the recorded spectra. Addition of organic solvents reduces the magnitude of the lipid enhancement phenomenon by raising the level of fluorescence in the non-enhanced solution. A methanol concentration of around 40% (v/v) is the maximum amount which will still allow a useful enhancement effect to be observed.

Detector system

An aqueous solution is necessary for the fluorescence enhancement of ANS in the presence of polar lipids, while chromatography of these compounds appears best accomplished in organic mobile phases[13]. For this reason, chromatography was carried out with methanol as the mobile phase, with subsequent addition of aqueous ANS to the column effluent for detection.

The experimental set-up which accomplishes this goal is shown in Fig. 3. The mobile phase is delivered by a syringe pump (Series 4100; Varian Aerograph, Walnut Creek, Calif., U.S.A.) to the chromatographic column. The column consists of ammonium hydroxide-treated Corasil II (Waters Assoc., Milford, Mass., U.S.A.)[14] packed into a 90 cm × 2.1 mm I.D. seamless, stainless-steel tube terminated with a stainless-steel frit of 5-μm average porosity (Crawford, Cleveland, Ohio, U.S.A.). A high-pressure reciprocating piston pump (M 6000; Waters Assoc.) was employed to deliver a phosphate buffer solution of the fluorescent dye to the T-piece and mixing coil. An isolation coil was used to separate the chromatographic column from the T-piece. This was found necessary to prevent the ANS solution from backing up into the column during injection of samples.

Presumably, micelles are formed upon the elution of lipids from the column into the aqueous solution. ANS, which partitions into the nonpolar interior of the micelle, exhibits enhanced fluorescence and is detected by the fluorescence detector (Model 1209; Laboratory Data Control, Riviera Beach, Fla., U.S.A.). The excitation source of this detector is an UV lamp with broad emission from 300–400 nm peaking at 365 nm. A sharp UV cut-off filter allows transmission of the ANS fluorescence to the

photodetector while blocking excitation light. The broadband nature of the detector response minimizes effects of wavelength shifts in fluorescence emission.

Chromatographic measurements

Several detector variables were investigated by performing chromatography of standard polar lipids. Pure standards of phosphatidylcholine dipalmitoyl, cardiolipin, and sphingomyelin were obtained from Sigma (St. Louis, Mo., U.S.A.), and gangliosides were purchased from Analabs (North Haven, Conn., U.S.A.). A mobile phase of 1% ammonium hydroxide in methanol was used to minimize tailing of these very polar compounds. The mobile phase flow-rate of 0.33 ml/min was chosen as a reasonable compromise between chromatographic efficiency and analysis time. A flow-rate of 3.0 ml/min for the buffered ANS solution was found to be sufficient in diluting the mobile phase, while still allowing a high concentration of lipid for detection.

Physical interactions between the fluorescent dye and a lipid micelle are likely to depend on the conditions of the experiment such as temperature, pH, presence of various ions, other compounds, etc. Fig. 4 demonstrates the dependence of the detector response (peak heights) for standard lipids upon pH. While there is no great pH dependence, a switch from the ANS solution in phosphate buffer to pure water resulted in a substantially smaller response. No studies of the effects of temperature or various ions were performed.

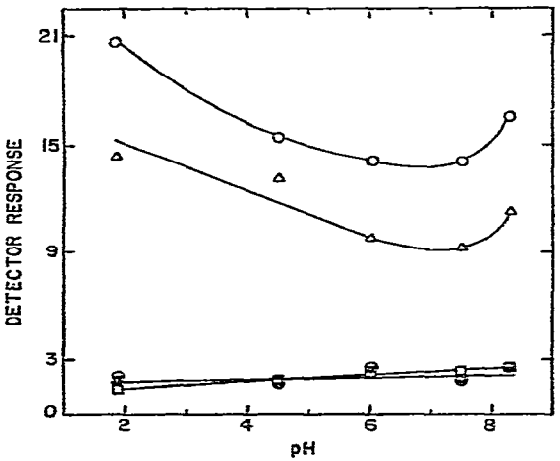

Fig. 4. Dependence of detector response on pH. Conditions: 1×10^{-3} *M* ANS in 0.1 *F* phosphate buffer; flow-rate, 3.0 ml/min. Data reflect the pH of solution actually entering the fluorescence detector. Detector responses were measured as relative peak heights of the solutes chromatographed (approximately 30 μg each). ○, Phosphatidylcholine; △, sphingomyelin; □, cardiolipin; ⊖, gangliosides.

The effect of ANS concentration on the detector response was also investigated. Fig. 5 illustrates the corresponding dependence using phosphatidylcholine as the standard solute. The optimum concentration of the fluorescent agent for maximum sensitivity under these conditions was 5×10^{-4} *M*. A possible explanation for the decreased response at high ANS concentration may be normal fluorescent self-quenching effects, or a disruption of the micelle by ANS.

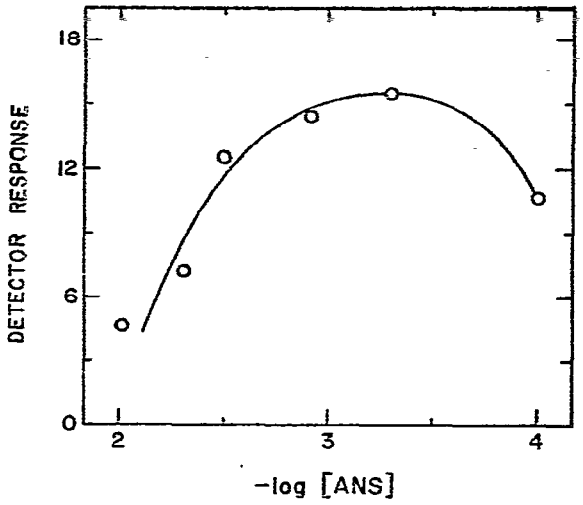

Fig. 5. Dependence of detector response on the concentration of ANS added through T-piece. Conditions: solute, 32 μg phosphatidylcholine; ANS was dissolved in 0.1 *F* ammonium phosphate buffer, pH 4.55.

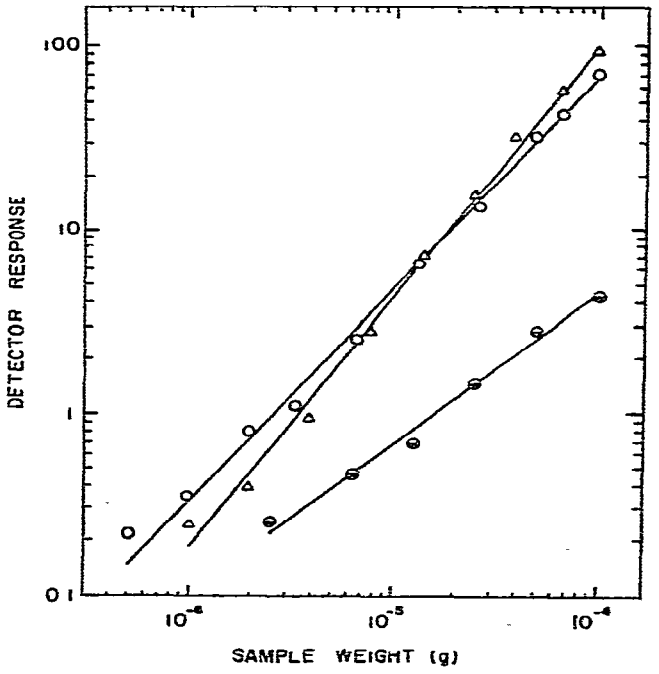

Fig. 6. Linearity of detector response for various standard solutes. Conditions: 5×10^{-4} *M* ANS dissolved in 9.4×10^{-2} *F* H_3PO_4 (pH 7.00). ○, Phosphatidylcholine; △, sphingomyelin; ⊖, gangliosides.

Fig. 6 demonstrates that different lipids yield different responses, but the relationship between peak height (detector response) and sample weight injected is essentially linear. The relation of response to solute structure has not been extensively studied as yet. However, the compounds which respond all have very polar head groups, and would be expected to form micelles easily.

A typical chromatogram of a mixture of standard phospholipids is shown in Fig. 7. It demonstrates that even with non-standard chromatographic conditions a useful separation is possible with the present system. A chromatogram of the polar lipids in a 1-ml Folch extract of human blood plasma[15] is shown in Fig. 8. No identification of the constituents of the plasma sample was carried out.

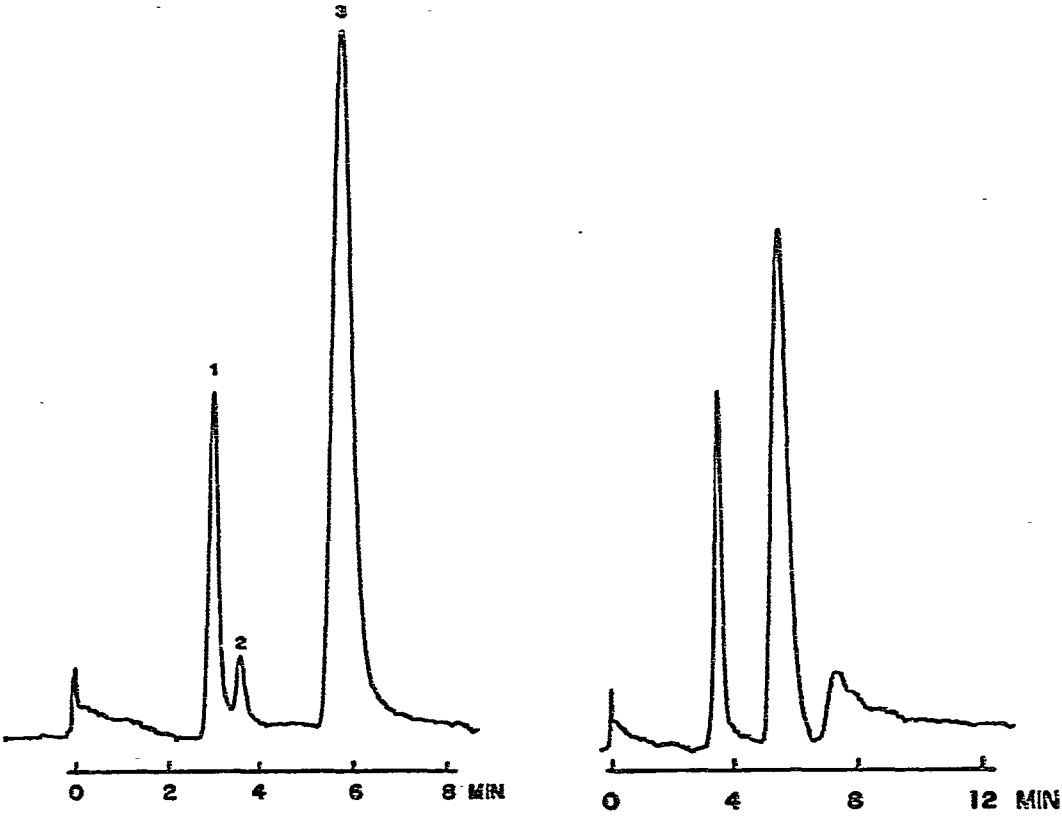

Fig. 7. Chromatogram of standard phospholipids. Conditions: mobile phase, 1% ammonium hydroxide in methanol; flow-rate, 0.33 ml/min; ANS solution, same as for Fig. 6. 1 = Cardiolipin (84 μg); 2 = solvent impurity; 3 = phosphatidylcholine (29 μg).

Fig. 8. Chromatogram of polar lipids of human plasma. Conditions, same as in Fig. 7.

DISCUSSION

The inherent sensitivity of fluorescence measurements will undoubtedly cause a wider use of LC fluorescence monitors in the near future for detection of both naturally fluorescent and selectively derivatized compounds. The detection principle described

in this work can serve as a valuable addition to high-sensitivity detection. Although it has thus far been demonstrated for specific cases, more systematic studies of fluorescent dyes and their properties should lead to further applications of this and related principles in the detection of other classes of compounds.

This work demonstrates that even at relatively non-optimized detection conditions, fluorescence enhancement detection has led to a significant improvement in sensitivity for polar lipids compared to hitherto available continuous detection systems. Whereas different lipids studied as model solutes in this work possess different (and yet, comparable) response factors, a linear relationship between detector response and solute concentration was maintained. A practical sensitivity limit for the system as constructed was approximately 500 ng of phosphatidylcholine. This amount corresponds to a detection limit of 17 ng/sec for a peak of 29-sec width at half-height.

As previously indicated, the present system is a compromise between chromatography and successful detection. This situation might be improved through the use of mobile phases containing tetrahydrofuran and dioxane which are less polar than methanol but are still water-miscible. Such solvents would ideally show minimal interference with the fluorescence enhancement effect while expanding the range of useful chromatographic separations. Another approach would be the use of a system analogous to the reversed-phase system described by Arvidson[16] for the separation of egg-yolk lecithins on alkylated Sephadex with a methanol–water mobile phase. This arrangement might change the chromatographic separation as well as the necessary amount of ANS buffer solution added at the end of the column. The large dilution (10:1) of column effluent currently necessary is one unfortunate sensitivity-reducing factor in the system, which might be improved by such a scheme. However, no mechanically stable analog of alkylated Sephadex suitable for high-pressure LC is available at present.

In future efforts to improve sensitivity it will be necessary to concentrate primarily on reducing the low-frequency baseline noise, which is the major limitation of the currently existing system. The main contributors to such noise are instabilities in solvent delivery by the pumps (pulses) and non-instantaneous mixing of the two solvent streams in the T-piece. Further refinement in design of the system should reduce such problems, and thus allow more significant utilization of the available sensitivity of this detection principle.

Another, but perhaps less attractive alternative to alleviating the problems of incompatibility of the mobile phase composition with detection conditions could be the design of a transport detection system, similar to that described by Werthessen *et al.*[17].

Finally, it should be emphasized that the fluorescence enhancement effect that forms the basis of detection shown in this work should be looked upon as an example of the many fluorescence-related phenomena[18] that can be potentially utilized in LC detection of non-fluorescent compounds.

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