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High-performance liquid chromatographic determination of acridine orange in nucleic acids isolated from dye-treated *Himantormia lugubris* thalli

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ABSTRACT

The natural fluorescence of acridine orange was used for detecting and determining this dye in extracts of lichen thalli floated on acridine solutions after reversed-phase chromatography on Nucleosil 5 C_8; acetonitrile–water (80:20) was used for elution at a flow-rate of 1.2 ml min^{-1}. Fluorescence emission was monitored at 460 nm using an Mn^{2+}–atranorin chelate as internal standard. The response was linear over the range 0.01–0.4 μg of injected dye. This method is superior to those published previously in terms of sensitivity (from 60 to 300 times higher) and rapidity. This method was applied to measure the amount of acridine orange that binds to polynucleotides in lichen samples and to establish that cyclic AMP is able to impede the binding of the dye to lichen DNA. The method could be applied to intercalating action studies on DNA and dye detection in biological samples.

INTRODUCTION

Acridine dyes act as intercalating agents in bacterial episomes, impeding both replication and transcription [1]. This action, in a similar way to catabolite repression effected by glucose [2], is reversed by cyclic AMP by protecting the formation of the transcription initiation complex [3]. In addition to those described for bacteria, several catabolite-sensitive promotors have been found in eukaryota. In these organisms, the catalytic subunit of A kinase is sufficient to induce expression of cyclic AMP-sensitive genes [4]. Many promoters in eukaryota are also sensitive to acridine dyes and, to our knowledge, to nalidixic acid, like many prokaryota operons [5,6]. Production of some enzymes involved in the synthesis of lichen phenolics is regulated by catabolite-sensitive promoters, also inhibited by acridine orange, in *Evernia prunastri* [7], *Pseudevernia furfuracea* [8] and *Himantormia lugubris* [9]. This inhibition is always reversed by cyclic AMP.

Acridines, usually acridine isothiocyanate [10], are often used as derivatizing agents, but the excess of dye is not easily destroyed and this excess produces, according to the technique, a corresponding spot or peak that must be identified. The most usual chromatographic procedures applied to acridine dyes are paper [11,12] and silica gel [13,14] and cellulose [15] thin-layer methods using chloroform or dimethylformamide–water (35:65, v/v), respectively, as mobile phase. Detection procedures include chromogenic reactions [11,12] and fluorescence under UV radiation. The determination of acridines after their elution from thin layers by using a conventional spectrofluorimeter is linear over the range 3–25 μg [15–17], but it is probable that even lower amounts of the dye could be intercalated in the polynucleotide sequence. Gas–liquid chromatography (GLC) using 5% (w/w) Triton X-305 on acid-washed Chromosorb G (70–80 mesh) [18] gives retention times longer than 40 min for acridine derivatives. However, mixtures of bound acridine with free nucleotides or extraction agents could produce many artifactual interferences during derivatization

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and during a prolonged separation. Hence an accurate, easy, rapid and sensitive method for determining acridine dyes is required to relate the amount of the intercalating agent in DNA and the degree of inhibition of the sensitive promoter. In addition, the method must be equally applicable to other biological samples in which traces of acridine accidentally occur.

In this paper, we report the determination of acridine orange by high-performance liquid chromatography (HPLC) either in standard solutions or in nucleic acid fractions isolated from *H. lugubris* thalli. As the amount of isolated nucleic acid from lichen thalli was always very small and contained very low amounts of bound acridine, the method needed to be sufficiently sensitive to determine the dye. The use of protamine to precipitate DNA introduces an additional problem because of the fluorescence of the peptide, but the very good resolution of the chromatographic separation avoids any errors.

EXPERIMENTAL

Plant material

Himantormia lugubris (Hue) Lamb, growing on soil in King George Island (Antarctica), was used. Thalli were air-dried and stored in the dark at 5°C until required.

Sample preparation from incubated thalli

Samples of 0.5 g of air-dried thalli were floated on 12 ml of 0.1 *M* sodium acetate–acetic acid buffer (pH 6.8) for 3 h at 26°C in the dark. When indicated, 0.1 m*M* acridine orange and 0.5 m*M* cyclic AMP were added to the buffer. After incubation, thallus samples were washed with distilled water, gently dried with filter-paper and macerated in a mortar with 25 ml of chloroform for 15 min at room temperature to extract lichen phenolics [9]. Homogenates were filtered through Whatman No. 3 filter-paper and solid residues were air dried and stored at −34°C. Filtrates were dried in an air flow and residues were redissolved in 1.0 ml of acetonitrile (HPLC grade) and filtered through Millipore GS filters (0.22 μm pore diameter). These filtrates are called chloroformic extracts.

Thalline powders were macerated with 4.0 ml of distilled water and centrifuged at 38 000 *g* for 30 min at 2°C. The supernatants were adjusted to 5% (w/v) protamine sulphate and stored for 20 min in ice. Then, they were spun at 43 000 *g* for 30 min at 0°C. The pellets were washed with 2.0 ml of dilute protamine sulphate solution (0.05%, w/v), extracted with 2.0 ml of acetonitrile (HPLC grade) and filtered through Millipore GS filter as above. These filtrates are called nucleic acid fractions.

Reagents

Acridine orange, atranorin, cyclic AMP and protamine sulphate were obtained from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade) (Carlo Erba, Milan, Italy) was used as received and doubly distilled water (Carlo Erba) was filtered through Millipore GS filters (0.22 μm pore diameter) before use. A Nucleosil 5 C_8 column (Varian, Palo Alto, CA, USA) was kept in acetonitrile until required.

HPLC separation of acridine orange

HPLC was performed on a Varian Model 5060 liquid chromatograph equipped with a Varichrom TM VUV 10 V detector and a Fluorichrom TM detector (Varian) in series and a Vista CDS 401 computer. The chromatographic conditions were as follows: column, Nucleosil 5 C_8 (125 mm × 4 mm I.D.); sample loading, 10 μl; mobile phase, acetonitrile–water (80:20, v/v), isocratic; flow-rate, 1.2 ml min^{-1}, as deduced from the Van Deemter equation; temperature, 20°C; detectors, UV (254 nm for atranorin), VIS (440 nm for Mn^{2+}–atranorin chelate complex and 490 nm for acridine orange) and fluorescence (excitation wavelengths from 340 to 380 nm using filters No. 7-54 and 7-60 from Varian, and emission wavelength 460 nm using filters No. 3-71 and 4-76 from Varian); 0.002 a.u.f.s.; attenuation, 64; internal standard, 1.0 mg ml^{-1} atranorin as Mn^{2+} chelate.

Chelate preparation

To 2.0 ml of 3.0 m*M* atranorin solution in acetonitrile, 1.0 ml of an aqueous solution of $MgCl_2$ or $MnCl_2$ (50 m*M*) was added [19]. The mixtures were incubated for 1 h at 30°C and atranorin was extracted with 6.0 ml of diethyl ether after vigorous shaking for 2 h to separate the phenolic derivative from the excess of inorganic ions [20]. The organic phases were dried *in vacuo* and used as internal standards.

Absorbance and fluorescence emission spectra were obtained by using a Varian DMS 90 spectrophotometer and a Kontron (Milan, Italy), SFM 25 spectrofluorimeter, respectively.

RESULTS AND DISCUSSION

Choice of detector and internal standard

As 0.1 m*M* acridine orange in acetonitrile showed a natural, pale green fluorescence, a fluorimetric detector (Fluorichrom TM, cell volume 12.5 μl) was used. Atranorin was chosen as an internal standard for several reasons. It is the main component of the phenolic fraction of many lichen species [21], including *H. lugubris* [9], it produces natural fluorescence [22] and it does not change substantially the pH of acridine solutions (water-dissolved acridine orange has a pH of 5.46).

Spectra of the fluorescence emission of atranorin and its chelates are shown in Fig. 1. However, atranorin in acetonitrile produced a quantitative response in the absorbance detector at 254 nm (Fig. 2A), with a retention time of 3.01 min, but no significant fluorescence response was found (Fig. 2B). Atranorin in lichen thalli occurs as a salt or chelate with inorganic cations [22,23]. Hence both magnesium and manganese chelates were prepared and chromatographed under the same conditions. The retention time of the absorbance peak at 440 nm for the Mn^{2+}–atranorin complex (Fig. 2C) coincided with the fluorescence peak, but decreased to 1.34 min from that for free atranorin (Fig. 2D).

By using this chelate as an internal standard, the retention time of which slightly increased to 1.41 min, acridine orange separated as a well resolved peak with a retention time of 10.54 min. The fluorescence response was 2.5 times higher than that obtained in the absorbance detector at 490 nm (Fig. 2E and F). A very small peak at 9.37 min seemed to be due to a contaminant of the standard dye. As the retention time in GLC for acridine varied from 40 to 50 min on Triton X-307–Chromosob G [17]. The HPLC separation described here was 4–5 times more rapid than GLC.

Baseline correction was always applied. This was constructed from the start of the first peak (internal standard) to the lowest valley point at the end of the same peak and, in this way, after a baseline segment was constructed, the area of the peak was corrected. The result was stored in a time and area file and then the next baseline segment was calculated for the included peak. Each successive baseline segment started at the end of the preceding one, to include all the peaks [24].

Linearity of response

Once the detector conditions had been chosen, the flow-rate was changed from 0.2 to 2.0 ml min^{-1}

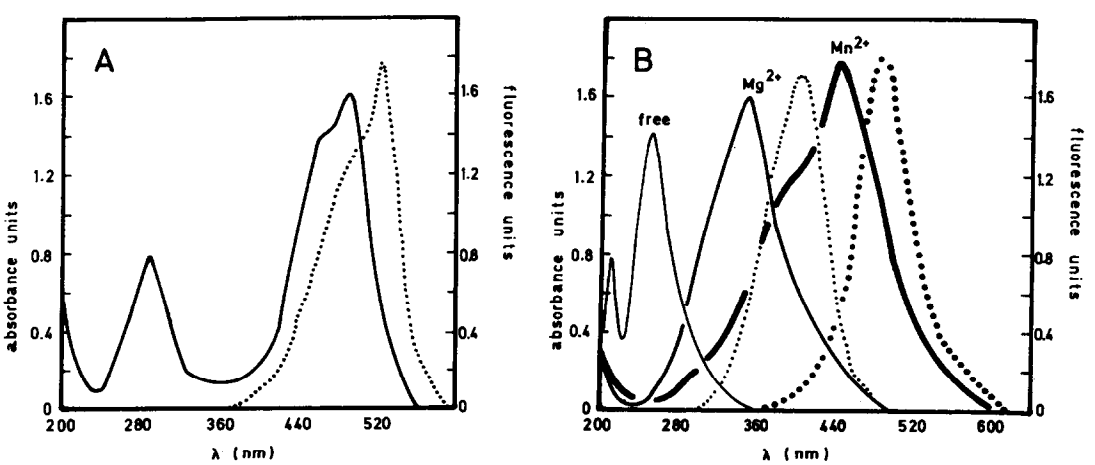

Fig. 1. Absorbance (continuous lines) and fluorescence emission (dotted lines) of (A) acridine orange and (B) atranorin as free or chelate compound.

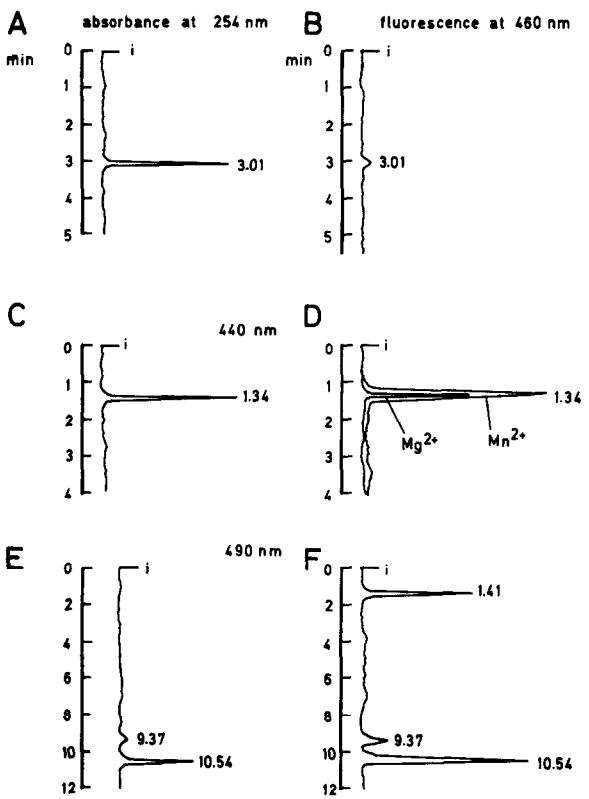

Fig. 2. HPLC traces of free atranorin monitored by (A) its absorbance at 254 nm or (B) fluorescence emission at 460 nm, Mg^{2+} and Mn^{2+} chelates of atranorin monitored by (C) their absorbance at 440 nm, where both peaks are exactly superimposed, or (D) fluorescence emission at 460 nm, and acridine orange monitored by (E) its absorbance at 490 nm or (F) fluorescence emission at 460 nm using Mn^{2+}–atranorin chelate as internal standard. The numbers on the peaks indicate retention times (min) and i indicates injection.

and the number of effective theoretical plates was calculated. The maximum number of theoretical plates (750 mm^{-1}) was obtained for a flow-rate of 1.2 ml min^{-1}. This flow-rate was then adapted throughout. The response of the detector was virtually linear in the range 0.01–0.4 μg injected (Fig. 3). The error inherent in direct calibration was estimated as the standard error for six repeated injections for each concentration of standard solutions. The equation for the calibration straight line was obtained by linear regression, the r^2 value of which measured the goodness of fit.

Determination of acridine orange in chloroformic extracts of H. lugubris

Samples of 0.5 g of *H. lugubris* thalli were floated on 0.1 m*M* acridine orange in 0.1 *M* acetate buffer (pH 6.8) for 2 h in the dark at 26°C or, alternatively, on 0.1 *M* acetate alone, using the latter as a culture control. Chloroformic extracts of both samples were dried *in vacuo* and residues were redissolved in 2.0 ml of pure acetonitrile. The chromatographic traces indicated that only atranorin gave a fluorescence peak at 1.41 min, as barbatolic acid, the other phenolic contained in this extract, did not show natural fluorescence (Fig. 4A). When extracts from samples floated on acridine orange were chromatographed in the same way, a sharp, well resolved peak appeared with a retention time of about 10.6 min (Fig. 4B). This peak increased when 1.0 μg acridine orange was added to the extracts dissolved in acetonitrile (Fig. 4C).

Determination of acridine orange in the nucleic acid fraction from H. lugubris

The precipitate of total nucleic acids, obtained by treatment with 5% (w/v) protamine sulphate, was either dialysed against distilled water or filtered through a column of Sephadex G-75 (10 cm × 1 cm I.D.), using distilled water as the mobile phase. In both instances, acridine orange was lost during these processes. Even during filtration through Sephadex G-75, visible fluorescence was retained in the column whereas nucleic acids were eluted in the void volume. Either acridine orange was removed from nucleic acids by physical strength or was exchanged from DNA to protamine, it did not seem convenient to remove protamine sulphate from the precipitate. Therefore, the complete pellet was washed with dilute protamine sulphate solution (0.05%, w/v) and extracted with 2.0 ml of pure acetonitrile prior to chromatography. Extraction of the dye from nucleic acids was required in order to avoid the irreversible quenching of acridine fluorescence by guanine–cytosine pairs after binding of the dye to polynucleotides [25]. When acridine-untreated thalli were used as a control to precipitate nucleic acids, protamine gave a strong fluorescence peak with a retention time of 2.03 min (Fig. 5A) that did not completely overlap that of atranorin, used as an internal standard (1.41 min), and that increased after loading the sample with protamine

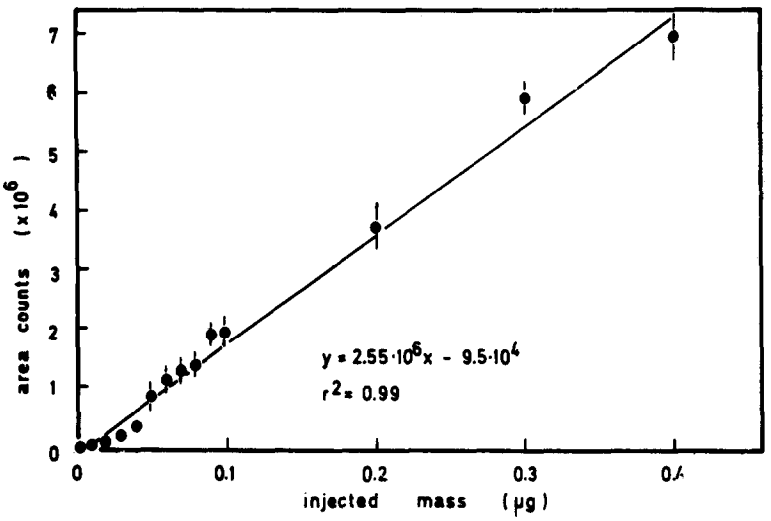

Fig. 3. Direct calibration for acridine orange where area counts recorded for fluorescence emission behave as a linear function of the mass injected. Values are the means of six replicates. Vertical bars give standard errors where larger than the symbols.

itself (Fig. 5B). A short but well resolved peak with a retention time of about 10.6 min (Fig. 5C), which increased on adding 1.0 μg of acridine orange to the sample (Fig. 5D), revealed the appearance of the dye extracted from the pellet of nucleic acids.

Application of the method to determine the role of cyclic AMP on the intercalation process

This HPLC method was applied to determine the time course of acridine orange intercalation in nucleic acids, mainly DNA [1,2], and the role of cyclic

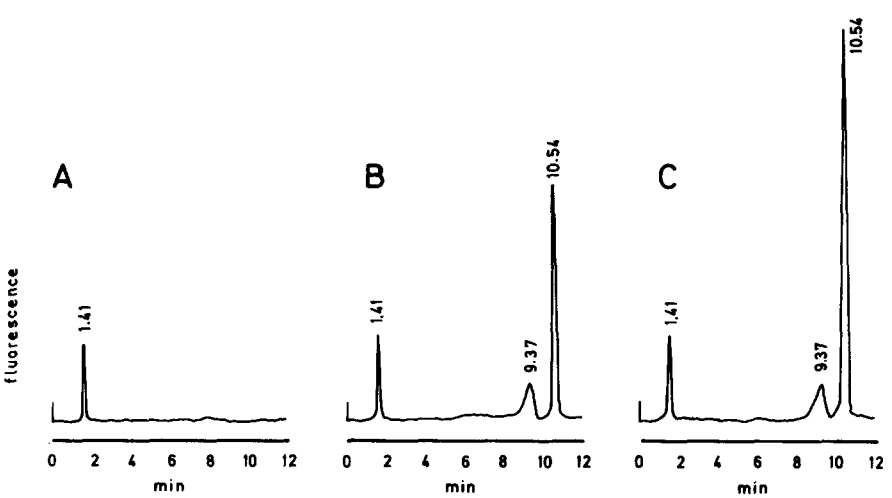

Fig. 4. HPLC identification of acridine orange extracted with chloroform from thalli of *H. lugubris* floated for 3 h on 0.1 m*M* acridine orange in 0.1 *M* acetate buffer (pH 6.8). (A) Control extract obtained from untreated thalli; (B) extract from thalli incubated on the dye; (C) extract used in (B) loaded with 1.0 μg of acridine orange in the injection volume.

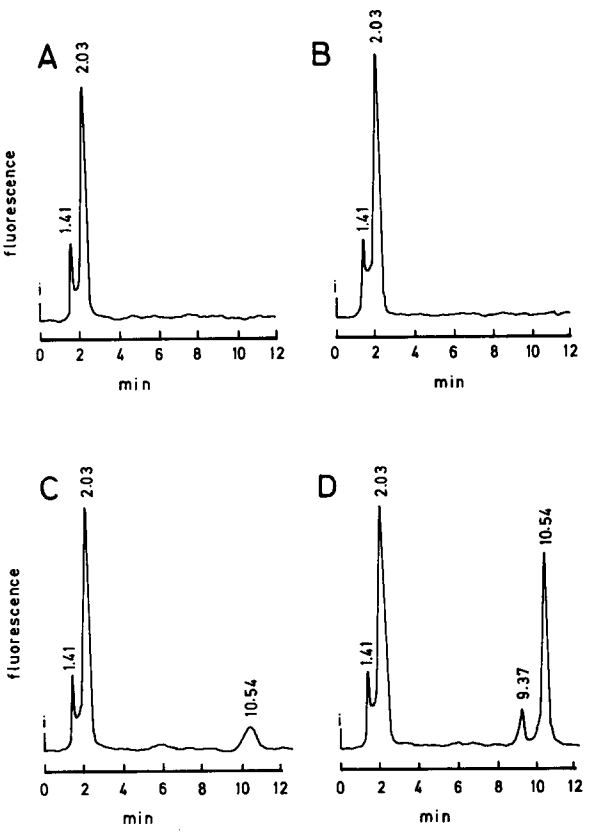

Fig. 5. HPLC of acridine orange bound to nucleic acids after precipitation with protamine sulphate. (A) Dye-untreated thalli used as control; (B) the same extract loaded with pure protamine sulphate; (C) extract prepared from thalli floated for 3 h on 0.1 m*M* acridine orange; (D) the same extract loaded with the dye.

AMP in this process. Samples were floated for 3 h in the dark on 0.1 *M* acetate containing 0.1 m*M* acridine orange or 0.1 m*M* acridine orange and 0.5 m*M* cyclic AMP. Both chloroformic extracts and protamine precipitates were prepared as indicated and redissolved in 2.0 ml of acetonitrile prior to chromatography. Fig. 6A shows the time course of acridine orange in chloroformic extracts. The amount of the dye increased continuously with the time of thalli incubation. When cyclic AMP was included in the incubation medium, the amounts of the dye recovered in the extracts were always higher than those found in the absence of the nucleotide, although the maximum value was obtained at 1 h of thalli incubation. When both dye and cyclic AMP were included in the incubation medium, the amount of acridine orange bound to the nucleic acid fraction was always lower than that extracted from thalli floated on the dye alone (Fig. 6B). These results, which were found to be highly reproducible, are in agreement with those obtained for binding of acridine to isolated DNA by using conventional spectrofluorimetric methods [26].

As cyclic AMP impedes the binding of acridine orange to the polynucleotide fraction (the excess of acridine, which remained as free dye after thalli incubation on the cyclic nucleotide, was recovered in the chloroformic extract), this action would be in agreement with that proposed by Ullmann [27] for the function of catabolite-sensitive operons during

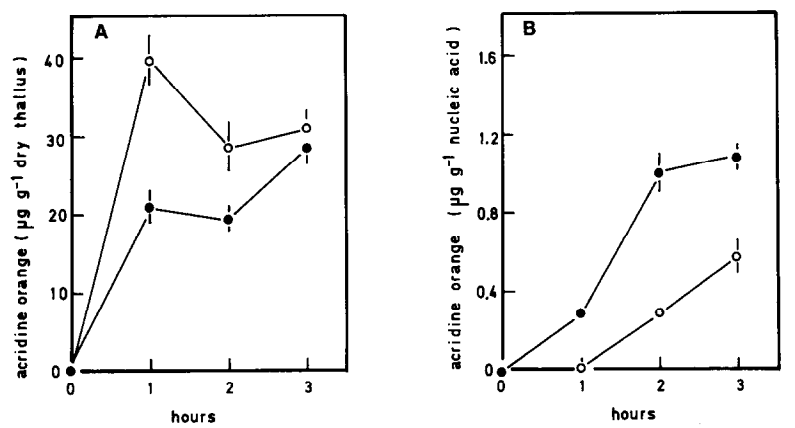

Fig. 6. (A) Time course of acridine orange accumulation by thalli of *H. lugubris* floated on (●) 0.1 m*M* dye or (○) 0.1 m*M* dye and 0.5 m*M* cyclic AMP. (B) Time course of the binding of acridine orange to nucleic acids from *H. lugubris* thalli floated on (●) 0.1 m*M* dye or (○) 0.1 m*M* dye and 0.5 m*M* cyclic AMP. Values are the means of four replicates. Vertical bars give standard errors where larger than the symbols.

experimental inhibition, even for cells in which cyclic AMP was not really produced.

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