

## Note

---

### Chromatography of Astra Blue dyes with eluents containing potassium thiocyanate in organic solvents

DANILO CORRADINI and CSABA HORVÁTH\*

*Department of Chemical Engineering, Yale University, New Haven, CT 06520 (U.S.A.)*

(Received November 28th, 1984)

Astra Blue is a cationic phthalocyanine dye widely used in histochemistry for staining acidic polysaccharides and glycosamino glycuronans without interference from nucleic acid<sup>1,2</sup>. In the present study the chromatographic behavior of Astra Blue free base and quaternized Astra Blue was investigated by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) with octadecyl-silica as the stationary phase. With hydro-organic or neat organic mobile phases ordinarily employed in reversed-phase chromatography both dyes were very strongly retained on all stationary phases at column temperatures ranging from 0°C to 60°C. We found however that they could be eluted by using solutions of potassium thiocyanate in acetonitrile or methanol as the mobile phase. The chromatographic results suggest that none of the dye samples were homogeneous but contained more than one species that underwent some chemical transformation during the chromatographic run. The assumption of on-column reaction is supported by observation that upon lowering the column temperature the number of peaks increased and peak shape improved.

#### EXPERIMENTAL

Acetonitrile containing 0.6 M potassium thiocyanate was used to prepare sample solutions of 1 mg/ml which were passed through a 0.45- $\mu$ m membrane filter (Millipore, Bedford, MA, U.S.A.). The sample solutions showed no change in chromatographic and spectral properties after storage at 4°C over three months. In TLC experiments MK C<sub>18</sub> F (7.5 × 2.5 cm) reversed-phase plates from Whatman (Clifton, NJ, U.S.A.) were used. Two 5- $\mu$ l samples were spotted on each plate at a distance of 0.8 cm from each other and from the bottom. Ascending development to a height of 5-6 cm was normally complete in 5-8 min.

In HPLC experiments a Kratos (Ramsey, NJ, U.S.A.) Spectroflow 400 solvent delivery system and a Spectroflow Monitor SF 770 variable wavelength UV detector equipped with a Model MM 701 memory module were used with a Rheodyne (Berkeley, CA, U.S.A.) Model 712 injector valve having a 20- $\mu$ l sample loop. The chromatograms were obtained with an LDC (Riviera Beach, FL, U.S.A.) Model C-10 integrator. Column, injector valve and mobile phase reservoir were kept thermostatted by immersion in a Brinkman (Westbury, NY, U.S.A.) Model Lauda WB-

20/R constant-temperature bath. The column ( $150 \times 4.6$  mm) was packed with  $5 \mu\text{m}$  octadecyl-silica which was prepared from Spherisorb (Phase Sep, Norwalk, CT, U.S.A.) in our laboratory according to the procedure of Kováts and Boksányi<sup>3</sup> and had a carbon load of 11.8% (w/w). SynChropak Propyl 6.5 stationary phase was obtained from SynChrom (Linden, IN, U.S.A.) and slurry packed to obtain a  $150 \times 4.6$  column. A Beckman (Palo Alto, CA, U.S.A.) Model 25 double beam spectrophotometer was also employed in certain experiments. Reagent-grade chemicals from Fisher (Pittsburgh, PA, U.S.A.) were used exclusively and distilled water was obtained with a Barnstead distilling unit in our laboratory.

#### RESULTS AND DISCUSSION

The retention behavior of two Astra Blue dyes was first investigated in scouting experiments by TLC. None of the dyes eluted with neat methanol, acetonitrile or tetrahydrofuran or with their hydro-organic mixtures at widely different composition ranges. Neither would the dyes be eluted on regular silica gel plates by any of the neat organic solvents. On the other hand with saturated solutions of potassium thiocyanate in acetonitrile and methanol which contained 0.8 and 2.0 *M* potassium thiocyanate, respectively, both dyes eluted unretained. Upon reducing the concentration of potassium thiocyanate in the neat organic eluent a decreasing fraction of the sample still reached the solvent front but the bulk of the dye formed long streaks on the plates. Further lowering the potassium thiocyanate concentration more and more of

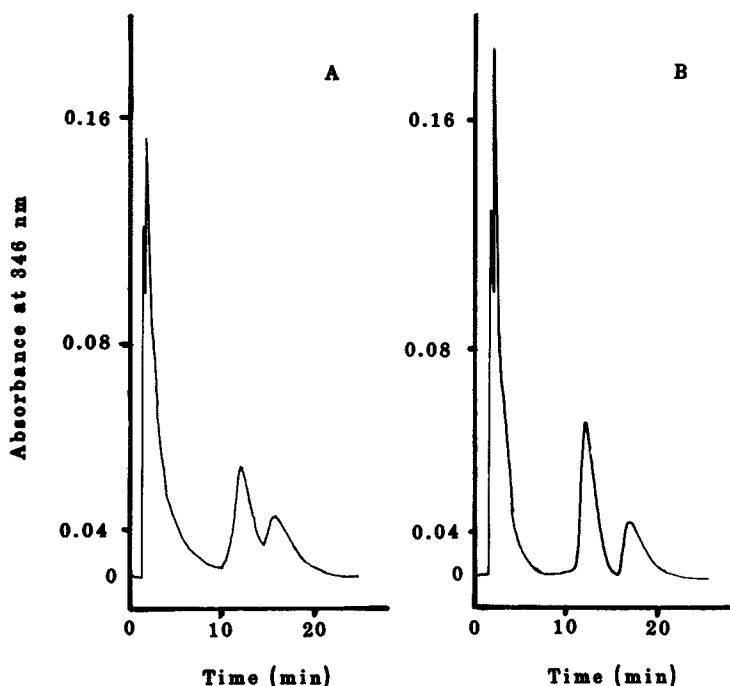


Fig. 1. Chromatogram of quaternized Astra Blue (A) and Astra Blue free base (B). Column,  $5 \mu\text{m}$  octadecyl-Spherisorb,  $150 \times 4.6$  mm; flow-rate, 1.0 ml/min; temp.,  $0^\circ\text{C}$ ; sample volume,  $10 \mu\text{l}$ .

the sample remained at the starting point and less and less of the dye formed streaks. Both dyes gave pronounced spots when 0.76 *M* potassium thiocyanate in acetonitrile–water (2:1), pH 2.5, was used as the eluent. Astra Blue free base yielded three spots having  $R_F$  values of 0.93, 0.83 and 0.69 whereas quaternized Astra Blue gave two spots having  $R_F$  values of 0.67 and 0.68. Nevertheless streaking occurred also under such conditions so that the total amounts of the samples applied were not present in the spots.

Neither in HPLC experiments could the two dyes be eluted from the column with neat acetonitrile, methanol or aqueous mixtures of these solvents in the temperature range from 0°C to 60°C. Similar observation was made with a variety of alkyl-silica columns used in reversed phase chromatography and with SynChropak Propyl column used in hydrophobic interaction chromatography<sup>4</sup>.

However acetonitrile containing 0.6 *M* potassium thiocyanate eluted at room temperature each dye as single peak having a retention factor of about 0.3. Upon decreasing the column temperature to 0°C and using the above mobile phase we obtained four peaks for each dye sample as shown on the chromatogram on Fig. 1, and their retention factors were 0.3, 0.6, 7.9 and 11.7 as measured at the apex. Upon increasing the column temperature the retention factors decreased and the Van't Hoff plot for the more retained peaks was linear. Whereas the retention of both dyes was similar on octadecyl-silica under such condition the peaks obtained from the free base were narrower and more symmetrical than those obtained from quaternized Astra Blue at all temperatures. Changing the flow-rate in the range from 0.5 to 2.0 ml/min had no effect on the retention factors and peak shape. The concentration of potassium thiocyanate in acetonitrile was varied from 0.6 to 0.1 *M* in a set of experiments at 1.8°C. With decreasing potassium thiocyanate concentration both retention and band broadening increased without improvement in resolution. Therefore it was not practical to lower potassium thiocyanate concentration below 0.6.

Experiments performed under conditions described above but by using methanol instead of acetonitrile yielded significantly lower retention values and inferior resolution. No improvement was observed upon increasing the concentration of potassium thiocyanate in methanol. All peaks showed the same absorption spectra in the range from 400 to 300 nm as measured by scanning at stopped flow and they were identical to those of the samples proper. The recovery of the dyes in the column effluent was measured and found to be nearly complete, therefore no significant fraction of any of the dye samples remained in the column.

There are some caveats regarding the use of eluents containing potassium thiocyanate. Their optical density is relatively high, *e.g.*, the cutoff of a 0.6 *M* potassium thiocyanate solution in acetonitrile is 280 nm with acetonitrile as the reference. At room temperatures the solutions turn yellow after 24 h. *Ad hoc* preparation of the eluent and storage in the refrigerator is recommended. Furthermore aqueous potassium thiocyanate can attack stainless steel and red colored ferric thiocyanate can further increase the optical density of the eluent. It is advisable to purge the chromatographic system after use of such eluent.

Nevertheless in HPLC of colored substances such as certain dyestuffs which cannot be eluted by ordinary eluents the use of potassium thiocyanate could be advantageous. Many basic dyes may strongly bind to the stationary phase via both electrostatic and hydrophobic interactions. An eluent comprising of potassium thio-

cyanate and an organic solvent appears to attenuate both kinds of interaction. Whereas organic solvents effectively reduce hydrophobic interaction, salts have the opposite effect above a certain concentration by virtue of increasing surface tension<sup>5</sup>. Unlike other salts, potassium thiocyanate has the unique property of not to increase the surface tension of water and relatively high solubility in certain organic solvents<sup>6</sup>. Yet it is an electrolyte and is expected to suppress electrostatic interactions. This may explain that the combination of potassium thiocyanate and organic solvent yield an eluent of unusual strength for certain applications where conventional eluents fail.

#### ACKNOWLEDGEMENT

This work was supported by grants No. GM 20993 and CA 21948 from the National Institute of Health, U.S. Public Health and Human Services.

#### REFERENCES

- 1 J. E. Scott, *J. Microbiol.*, 119(3) (1980) 373.
- 2 J. E. Scott and E. W. Hughes, *J. Microbiol.*, 129(2) (1983) 209.
- 3 E. sz. Kováts and L. Boksányi, *Ger. Pat.*, 29,305 (1979).
- 4 D. L. Gooding, M. N. Schmuck and K. M. Gooding, *J. Chromatogr.*, 296 (1984) 107.
- 5 W. Melander and Cs. Horváth, *Arch. Biochem. Biophys.*, 183 (1977) 200.
- 6 J. F. Coetzee and J. J. Campion, *J. Am. Chem. Soc.*, 89 (1967) 2517.