Journal of Chromatography, 268 (1983) 347–351
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,005

Note

High-performance liquid chromatography of distamycin A and its primary decomposition products as well as some synthetic analogues

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(Received May 24th, 1983)

Distamycin A (DA) (Fig. 1, R_1, R_2 and R_3 = CH_3, R_4 and R_5 = H) is an antibiotic with antiviral and oncolytic properties, first isolated and synthesized by Arcamone and co-workers[1,2]. In the course of some synthetic work in our laboratory, aiming at designing a flexible route to the substance itself[3] as well as to some analogues[4], we needed information on the stability of distamycin A under various conditions, used in its synthesis, purification and assay. This note reports simple high-performance liquid chromatographic (HPLC) systems for monitoring the purity of the antibiotic as well as synthetic analogues, modified either on one or more of the pyrrole nitrogens or on the amidine function in the side chain, together with results regarding the stability of such compounds in aqueous solutions at a moderately high pH, when the amidine function starts to undergo hydrolysis. Although the analogues are very closely related structurally, several, but not all of them, could be separated from each other, simultaneously giving an indication of the efficiency of HPLC and present limitations, when applied to compounds of this complexity.

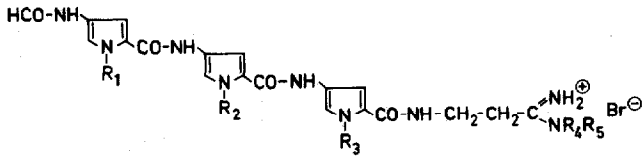

Fig. 1. Structure of DA and analogues.

MATERIALS AND METHODS

The distamycin A and analogues used originated from our earlier work[4]. Their high purity was confirmed in the present work.

Authentic distamycin amide was synthesized by analogy with DA[3] using β-aminopropionamide instead of the corresponding amidine as side chain-forming component. The ^1H nuclear magnetic resonance spectrum ($C^2H_3O^2H$) of the purified component resembled that of DA[4], especially as to the pyrrole signals. The aliphatic side-chain protons gave δ 3.55 and 2.50 ppm (triplets, 2H each), which clearly distinguishes it from DA.

0021-9673/83/$03.00

The mobile phases used for the reversed-phase separations consisted of phosphate buffers with ethanol as organic modifier. The buffers were prepared from orthophosphoric acid and sodium dihydrogen orthophosphate to an ionic strength of 0.1 *M*. The separations were run isocratically, both with and without pentane sulphonate present as counter ion. Pentane-1-sulphonic acid was obtained from Eastman-Kodak Co. (Rochester, NY, U.S.A.). All substances and solvents were of analytical or reagent grade.

Two different instrument set-ups were used, consisting of a Waters Assoc. Model 6000A or LDC Constametric Model III pump, a Waters Assoc. Model U6K or Rheodyne Model 7125 injector and a Waters Assoc. Model 450 or LDC Spectromonitor III detector. The detection wavelength was at 303 nm at high sensitivity.

The separation columns (150 × 4.6 mm) were packed by the balanced-density slurry technique[5] with Spherisorb C_{18} (5 μm) as chromatographic support for reversed-phase HPLC. The prepacked column (125 × 4.6 mm) filled with Spherisorb C_{18} (3 μm) and the bulk Spherisorb support were obtained from Phase Separations (Queensferry, Clwyd, U.K.). The separation columns were always preceded by a short guard column with Bondapak C_{18}/Corasil (Waters Assoc.). The separations were run under thermostatted conditions (25.0°C) using home-made column-block heaters.

RESULTS AND DISCUSSION

DA contains three pyrrole rings, four amide bonds and one strongly basic amidine function (see Fig. 1) and therefore has properties in common with basic peptides. The pyrrole rings confer hydrophobic properties to the compound, increasing its affinity for a reversed-phase support in comparison with hydrophilic peptides[6].

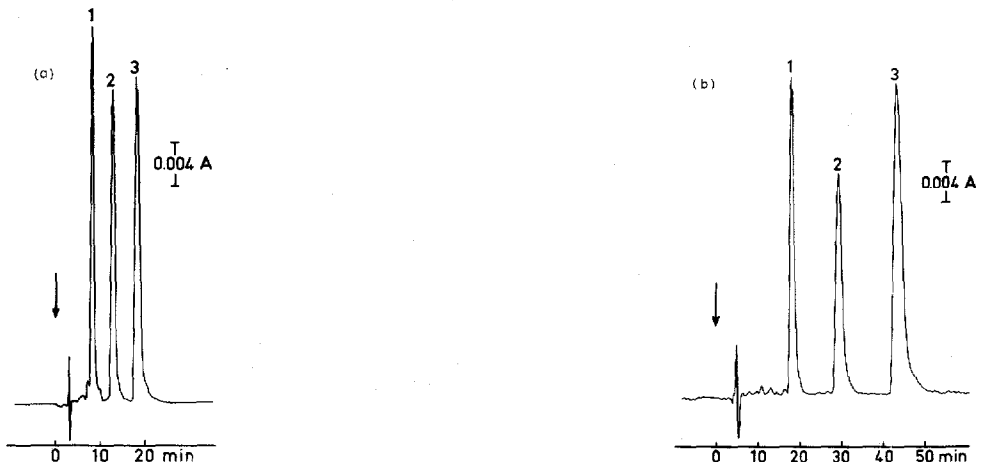

Fig. 2. Separation of 1-nordistamycin A (1; R_1, R_4 and R_5 = H, R_2 and R_3 = CH_3), distamycin A (2; R_1, R_2 and R_3 = CH_3, R_4 and R_5 = H) and 1-homodistamycin A (3; R_1 = C_2H_5, R_2 and R_3 = CH_3, R_4 and R_5 = H). (a) Mobile phase: phosphate buffer (pH 2.9)–ethanol (72:28); flow-rate, 0.5 ml/min. Support: Spherisorb C_{18} (5 μm, 150-mm column). (b) Compounds and conditions as in (a) except that the phosphate buffer was also 0.015 *M* in pentane-1-sulphonic acid.

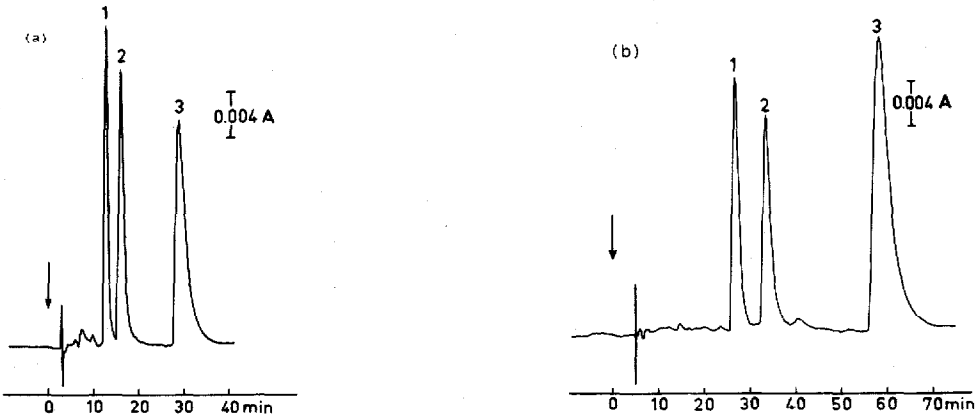

Fig. 3. Separation of DA (1; R_1, R_2 and R_3 = CH_3, R_4 and R_5 = H), its monomethyl (2; R_1, R_2, R_3 and R_4 = CH_3, R_5 = H) and dimethyl analogues (3; R_1, R_2, R_3, R_4 and R_5 = CH_3). (a) Chromatographic conditions as in Fig. 2a. (b) Compounds and conditions as in (a) except that the phosphate buffer was also 0.015 *M* in pentane-1-sulphonic acid.

Fig. 2a demonstrates separation of DA from two analogues, modified on the first pyrrole nitrogen. Peak 1 corresponds to 1-nordistamycin A and is somewhat more hydrophilic than the parent compound (peak 2). On the other hand, 1-homodistamycin A (peak 3) is, as expected, slightly more hydrophobic than the latter. Similarly, the 2-nor and 2-homo analogues and 3-nor and 3-homo analogues, respectively, could be separated from DA (results not shown). These experiments illustrate the application of a hydrophilic counter ion[7]. When a more hydrophobic ion-pairing agent, pentane-1-sulphonic acid, was used, the retention times increased approximately by a factor 2, as shown in Figure 2b. The order of elution was the same.

Figure 3a similarly illustrates separation of DA (peak 1) from its side chain monomethyl (peak 2) and dimethyl-substituted derivatives (peak 3) with phosphate as counter ion. The compounds elute in the order of increasing degree of substitution. The application of pentane-1-sulphonic acid as ion-pairing agent in this case, also increases the retention times approximately by a factor of 2 (Fig. 3b).

All analogues so far described could easily be separated from DA. On the other hand, Fig. 4 demonstrates separation of the three different nordistamycins and, as can be seen, this problem is a more exacting one. However, allowing considerably longer separation times, the application of a 3-μm support enables a satisfactory separation of the 2-nor analogue from the other isomers and a reasonable discrimination between the latter. Discrimination could, however, not be accomplished among the corresponding three homodistamycin A analogues, even on the 3-μm support and using the most extreme conditions with respect to flow-rate and separation time. This explains the results obtained when the 1-substituted analogues used in Fig. 2a and b were replaced by the corresponding 2- or 3-substituted derivatives mentioned above. As could be expected, the triethyl-substituted analogue (R_1, R_2 and R_3 = C_2H_5, R_4 and R_5 = H), owing to its higher hydrophobicity, gave rise to much longer retention times than did DA itself (up to three times longer under the conditions used in Fig. 2a and b).

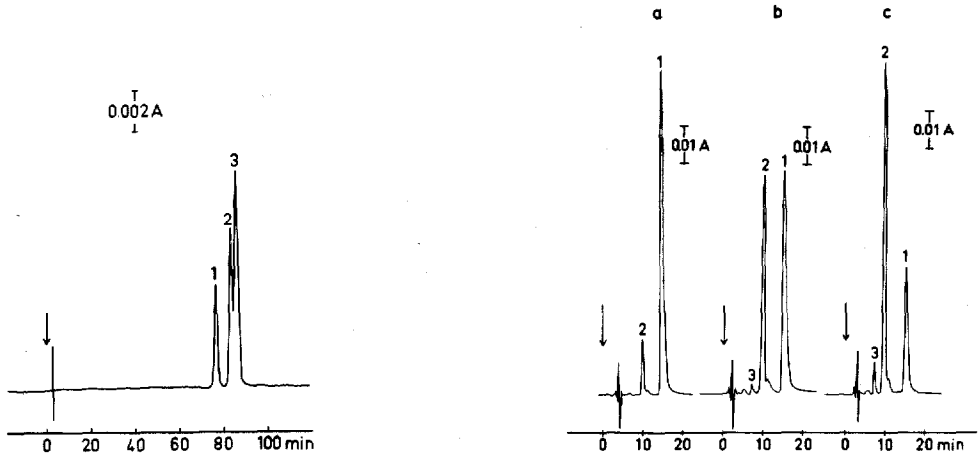

Fig. 4. Separation of 2-nordistamycin A (1; R_1 and R_3 = CH_3, R_2, R_4 and R_5 = H), 3-nordistamycin A (2; R_1 and R_2 = CH_3, R_3, R_4 and R_5 = H) and 1-nordistamycin A (3; R_1, R_4 and R_5 = H, R_2 and R_3 = CH_3). Mobile phase: phosphate buffer (pH 2.9)–ethanol (86:14), 0.01 *M* in triethylamine; flow-rate 0.5 ml/min. Support: Spherisorb ODS-2 (3 μm).

Fig. 5. Separation of DA and hydrolysis products after 2 h (a), 11 h (b) and 24 h (c) incubation in phosphate buffer (pH 10.7, μ = 0.1) at room temperature. Peak 1 refers to DA. Peak 2 behaves identically with authentic distamycin amide. All chromatographic parameters are as in Fig. 2a.

Aqueous solutions of DA are unstable[8], particularly at higher pH values. This instability is mainly due to the presence of the amidine function, since it is well known that this functional group is rapidly hydrolysed to the corresponding amide in alkaline media. It has also been shown previously that distamycin amide is the primary decomposition product, when DA is treated with aqueous sodium hydroxide for several hours[8].

In the stability test, DA was incubated with phosphate buffer at pH 10.7 at room temperature and Fig. 5 illustrates the course of the decomposition. Within 2 h, a new peak (2) appeared in the chromatogram. The position of this peak coincided with authentic distamycin amide. About half of the DA remained after 11 h and, as the reaction proceeded, a third peak (3) was observed. The same peak also emerged when authentic distamycin amide was incubated at this pH for several hours. We assume that this peak corresponds to the desformyl distamycin amide, as has been suggested earlier[8]. Preliminary experiments performed at lower pH values indicated that DA was much stabler under such conditions. Thus, at pH 9.0, more than 90% of the DA remained unchanged after 20 h. A solution of DA at pH 3 was found to be essentially stable when stored at room temperature for several days.

ACKNOWLEDGEMENTS

This study was made possible by research grants to U.R. from the National Swedish Board for Technical Development and the Swedish Natural Science Research Council.

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