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Separation and determination of polyether carboxylic antibiotics from *Streptomyces hygroscopicus* NRRL B 1865 by thin-layer chromatography with flame ionization detection

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ABSTRACT

Thin-layer chromatography coupled with flame ionization detection was used to develop a method to separate and to determine simultaneously three polyether carboxylic ionophore antibiotics (abierixin, nigericin and grisorixin) produced by *Streptomyces hygroscopicus* NRRL B 1865. Various proportions of chloroform, methanol and formic acid (or acetic acid as a substitute for formic acid) were used in the developing solvent to determine changes in R_F values of the antibiotics and to allow conditions for maximum resolution to be obtained. Development on Chromarods SII with chloroform–methanol–formic acid (97:4:0.6, v/v/v) gave satisfactory and reliable separations of the three polyether antibiotics. Under these conditions, the internal standard methyl desoxycholate was found to be suitable for their simultaneous determination in the lipid extracts of *Streptomyces hygroscopicus* NRRL B 1865.

INTRODUCTION

Carboxylic polyether ionophore antibiotics constitute a family of metabolites mainly produced by *Streptomyces* spp. Owing their anticoccidial and antimicrobial properties, they are used as feed additives to prevent coccidiosis in poultry [1] and to improve food efficiency and growth rate in sheep [2] and beef cattle [3].

These antibiotics have also been subjected to biosynthetic experiments [4]. In the course of our study on the biosynthesis of the carboxylic polyether ionophore antibiotic nigericin, produced by *Streptomyces hygroscopicus* NRRL B 1865 (Fig. 1), the addition of methyl oleate in the original fermentation culture increased the titre of the antibiotic pool [5]. Under these conditions, we isolated two nigericin closed-structural antibiotics which were produced at low levels, abierixin [5] and grisorixin [6] (Fig. 1); grisorixin was first isolated from *Streptomyces griseus* LAB 2142 [7].

In order to determine precisely the concentration of these three antibiotics in lipid extracts of *Streptomyces hygroscopicus* NRRL B 1865 and to measure the effects of the addition of effectors, a reliable and rapid method of analysis was required.

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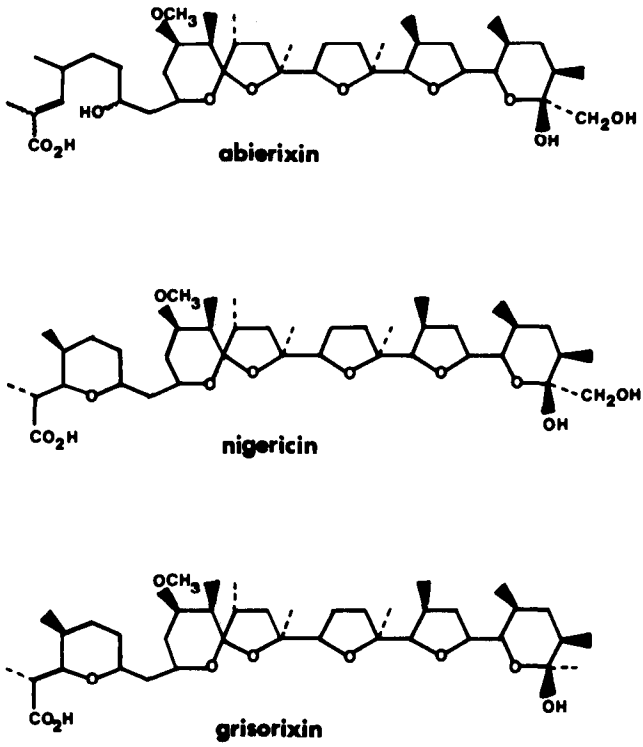

Fig. 1. Structures of three polyether carboxylic antibiotics produced by *Streptomyces hydroscopicus* NRRL B 1865.

Isolation by thin-layer chromatography (TLC) and detection with a vanillin–sulphuric spray [8] or by bioautography [9] were unsuitable for low levels of antibiotics. Complete separation and subsequent analysis of antibiotics has been achieved by high-performance liquid chromatography (HPLC), but this method is expensive and usually needs the preparation of fluorescent derivatives [10,11].

Separation on silica gel-coated quartz rods (Chromarods) by thin-layer chromatography and flame ionization detection (FID) was largely developed for qualitative analysis and the determination of plasma and tissue lipids [12]. The TLC–FID method appeared to be rapid, selective and sensitive, but had not previously been applied to antibiotic analysis, excepted for β-lactams and tetracycline [13], mainly because of the absence of an appropriate developing solvent. Therefore, the aim of this work was to establish the experimental conditions for analysis by TLC–FID to allow the accurate and reproducible separation and determination of abierixin, nigericin and grisorixin in lipid extracts of *Streptomyces hygroscopicus* NRRL B 1865.

EXPERIMENTAL

Antibiotic production

The three polyether carboxylic antibiotics abierixin, grisorixin and nigericin were produced by a strain of *Streptomyces hygroscopicus* NRRL B 1865. Cultures

were grown at 27°C for 7 days in a basal medium [5] with or without methyl oleate (1 g in 100 ml of medium) (Fluka, Buchs, Switzerland).

Antibiotic purification

A 15-l volume of the culture broth was filtered, yielding *ca.* 300 g of mycelial cake. After acidification at pH 4.0 with 1 *M* hydrochloric acid, the mycelial cake was homogenized in ethanol for 2 h in an Ultra-Turrax homogenizer. After 2 h, mycelium was removed by filtration and ethanol was eliminated by evaporation under vacuum, then the residual syrup which contained the antibiotics was solubilized in ethyl acetate–water (50:50, v/v). After drying over anhydrous sodium sulphate, the ethyl acetate extract was concentrated under reduced pressure and the residue was solubilized in chloroform. Antibiotics were fractionally separated by column liquid chromatography on a silica gel Type 60 (0.063–0.200 mm) (Merck, Darmstadt, Germany) column using cyclohexane–ethyl acetate with increasing amounts of ethyl acetate as eluting solvent [5].

Antibiotic analysis by TLC–FID

Antibiotic samples were chromatographed on silica gel Chromarods S-II and then analysed on an Iatroscan TH 10 Analyser, Mark III (Iatron Labs., Tokyo, Japan; French distributor, Delsi Instruments, Argenteuil, France) equipped with a flame ionization detector and connected to an Enica-10 electronic integrator (Delsi Instruments). The hydrogen flow-rate was 160 ml min^{-1} and the air flow-rate was 2 l min^{-1}. The scanning speed was 0.42 cm s^{-1}.

Chromarods were cleaned before use by soaking overnight in 5 *M* sulphuric acid, rinsed with distilled water, then dried at 110°C for 5 min and finally scanned twice in the Iatroscan just before use.

A chloroform solution containing equal amounts by weight (2μg/μl) of abierixin (A), nigericin (N) and grisorixin (G) was prepared to determine the effect of solvents on the resolution of antibiotics of Chromarods. For the purpose of standardization, a number of compounds including different antibiotics purified in our laboratory and lipids (Sigma, St. Louis, MO, USA) were tested. For the calculation of the response correction factors, three chloroform solutions were prepared; they contained a similar amount of methyl desoxycholate (5 μg/ml) (Sigma), which was used as the internal standard, and increasing amounts of each of the three antibiotics (2, 6 and 10 μg/ml).

For the determination of antibiotics from *Streptomyces hygroscopicus* NRRL B 1865, total lipids were extracted from 0.5 g of mycelia with chloroform–methanol (2:1, v/v) according the method of Folch *et al.* [14]. Methyl desoxycholate (1.25 mg) was added to the lipid extracts for the determination of the antibiotics in mycelia. Lipid solution in chloroform (20 mg/ml) was finally prepared for antibiotic analysis using TLC–FID.

For each of the aliquots, 0.5–1 μl of the chloroform solution was spotted on each of ten Chromarods held in an appropriate frame and transferred to the developing tank lined with filter-paper. Each antibiotic sample was analysed on 7–10 rods, the procedure being repeated at least once for each sample.

All solvent mixtures used for development were prepared by mixing x volumes of methanol with $100 - x$ (first trial, Fig. 2) or with $97 - x$ (second trial, Fig. 3)

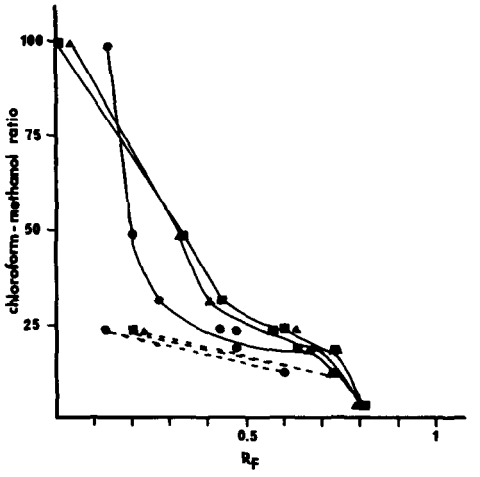

Fig. 2. R_F values of (●) abierixin, (■) nigericin and (▲) grisorixin plotted at various concentrations of chloroform, methanol and acetic acid (———, 0.5 vol.; ---, 1 vol.) in the developing solvent keeping the sum of the levels of chloroform and methanol constant (100) [chloroform–methanol–acetic acid = 100 − x : x : 0.5 (———) or 100 − x : x : 1 (----)].

volumes of chloroform. The organic acids (acetic and formic acids) were added to the 100 ml (Fig. 2) or 97 ml (Fig. 3) of solutions containing chloroform and methanol in amounts of 0.5–1 ml.

For the determination of antibiotics from *Streptomyces hygroscopicus* NRRL B 1865, the Chromarods were developed in chloroform–methanol–formic acid (97:4:0.6, v/v/v). The rods were then dried at 110°C for 5 min and transferred to the Iatroscan analyser for subsequent scanning.

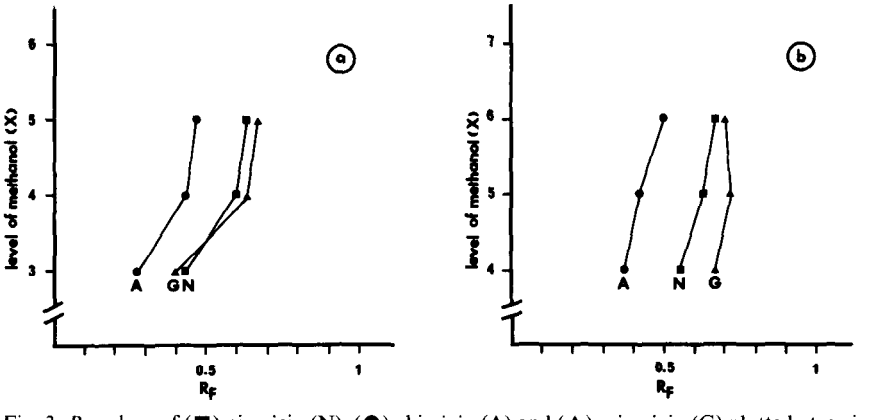

Fig. 3. R_F values of (■) nigericin (N), (●) abierixin (A) and (▲) grisorixin (G) plotted at various concentrations of chloroform, methanol and (a) acetic acid or (b) formic acid in the developing solvent. (a) The level of methanol (x) was varied while the level of acetic acid was kept constant (chloroform–methanol–acetic acid = 97:x:0.5). (b) The level of methanol (x) varied while the level of formic acid was kept constant (chloroform–methanol–formic acid = 97:x:0.5).

RESULTS AND DISCUSSION

Effects of solvents on the resolution of antibiotics

The chromatographic behaviour of a three-component standard mixture of antibiotics (grisorixin, abierixin and nigericin) on Chromarods was studied in order to determine suitable conditions for their separation and determination in biological samples by TLC–FID. Preliminary studies using chloroform–methanol as eluting solvent in different ratios such as that used in absorption TLC (95:5, v/v) [5] gave an inadequate separation of grisorixin and nigericin. Addition of a constant volume of acetic acid (0.5 vol.) improved the separation of the three antibiotics, especially with chloroform-to-methanol ratios between 20:1 and 30:1 (Fig. 2 and 3a), but no separation was observed with a larger proportion of acetic acid (1 vol.) whatever the chloroform-to-methanol ratio used (Fig. 2).

A developing solvent containing small amounts of methanol with formic acid (Fig. 3b) instead of acetic acid (Fig. 3a) [chloroform–methanol–formic acid (97:4:0.5, v/v/v)] increased specifically the relative mobility of grisorixin on Chromarods and therefore allowed a complete separation of the three antibiotics (Fig. 3b).

Concerning the analysis of biological lipid samples prepared from *Streptomyces hygroscopicus* NRRL B 1865, no interferences between the three antibiotics and the other lipid compounds were observed if the separation sequence was conducted with a developing solvent slightly enriched in formic acid (0.6 vol. rather than 0.5 vol.) [developing solvent:chloroform–methanol–formic acid (97:4:0.6, v/v/v)].

Internal standard for antibiotic determination

For the determination of antibiotics using the internal standardization procedure, relative mobilities of different lipid molecules were determined in order to select an appropriate internal standard with regard to its stability and solubility properties in both the extraction mixture and the chromatographic solvent and also its chromatographic behaviour.

Among the compounds tested, ionophore antibiotics (not produced by *Streptomyces hygroscopicus* NRRL B 1865) such as cezomicin [15], alborixin [16] and calcimycin [17], whose R_F value is similar to that of grisorixin (R_F = 0.67), and monensin [18], which overlaps with abierixin (R_F = 0.30), were not considered.

Similarly, phospholipids (phosphatidylethanolamine, phosphatidyl- and lysophosphatidylcholine) and glycolipids (mono- and digalactosyldiacylglycerols), which do not migrate from the origin, and non-phosphorus lipids (as free fatty acids, di- and triacylglycerols, fatty acid methyl esters, cholesterol esters, waxes), which migrate at the top with the developing solvent, (R_F >0.90), would interfere with endogenous bacterial lipids. Monoacylglycerol (1-monoheptadecanoin), which is virtually absent in lipid extracts of our strain, do not separate from nigericin (R_F = 0.56).

However, among the lipid biliary compounds tested, which included cholic acid, sodium tauro- and glycocholate (R_F = 0.10) and sodium desoxycholate (R_F = 0.30), methyl desoxycholate (MeDOC), which migrates (R_F = 0.47) between abierixin (R_F = 0.33) and nigericin (R_F = 0.57) (Fig. 4), was the most suitable internal standard for the determination of the antibiotics by TLC–FID. Moreover, no bacterial compounds were observed to display a similar R_F value to that of MeDOC.

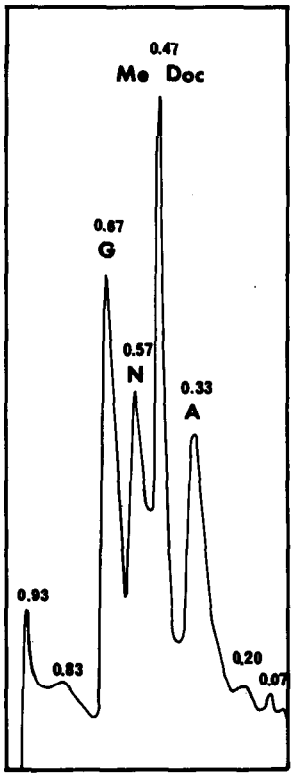

Fig. 4. TLC–FID of abierixin (A, 3 μg), nigerixin (N, 3 μg) and grisorixin (G, 3 μg) in chloroform solution (0.5 μl) with methyl desoxycholate (MeDoc, 1 μg) as internal standard. Separation was achieved on Chromarods SII with chloroform–methanol–formic acid (97:4:0.6, v/v/v) as the developing solvent.

Antibiotic calibration graphs

In order to calculate the amounts of individual antibiotics produced by *Streptomyces hygroscopicus* NRRL B 1865 in the mycelium or in the medium, standard solutions containing from 1 to 10 μg of antibiotic were analysed with the Iatroscan TH 10 analyser. Straight lines for peak-area ratio *versus* the weight ratio of the individual antibiotics to methyl desoxycholate wer obtained for abierixin, nigericin and grisorixin over the range considered (Fig. 5). Each point shown is the mean of five replicate analyses. The reproducibility of peak-area measurements depends on the amount of antibiotics applied. The relative standard deviation (R.S.D.) ranged from 2.3 to 6.1% intra-assay, and from 3.5 to 6.9% inter-assay when 2–10 μg of individual antibiotics were applied. In the same range of applied antibiotic, the reproducibility of the relative mobilities of the antibiotics was fairly constant and the R.S.D. averaged 0.5–1.5% for both intra- and inter-assay.

Determinations of FID responses using different amounts of antibiotics and normalized to that of 5 μg of methyl desoxycholate showed that, with 2–10 μg as the working range, the response factor was fairly constant (R.S.D. 2.1–5.6%) and similar for abierixin and grisorixin (0.4–0.5), which had the largest FID response, and the

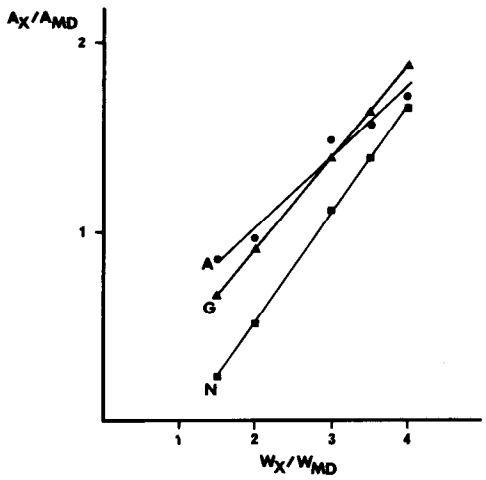

Fig. 5. Calibration lines of Iatroscan TLC–FID peak area (*A*) ratio *vs.* weight (*W*) ratio on the Chromarods SII for (●) abierixin, (■) nigericin and (▲) grisorixin as compared with methyl desoxycholate (MD) internal standard. Rods were developed in chloroform–methanol–formic acid (97:4:0.6, v/v/v).

yield was satisfactory (96.8 ± 3.5%). However, the response factor increased 1.5-fold with the amount of nigericin analysed (R.S.D. 3.1–8.5%) in the range 2–6 μg (yield 89.5 ± 8.6%) (Fig. 6). As for nigericin, similar correlations between response and amount of sample have been reported previously for neutral lipids (cholesterol, triacylglycerols) [19,20] and for phospholipids [20]. Hence, in order to improve the usefulness of TLC–FID for determination of the three antibiotics produced by *Streptomyces hygroscopicus* NRRL B 1865, it is recommended to limit the working range from 5 to 10 μg.

Determination of antibiotics in Streptomyces hygroscopicus NRRL B 1865

Determinations of antibiotics were performed on the lipid extracts of the mycelium of *Streptomyces hygroscopicus* NRRL B 1865 grown for 7 days on a basal medium with 1% methyl oleate. Only traces of grisorixin were observed in the myceli-

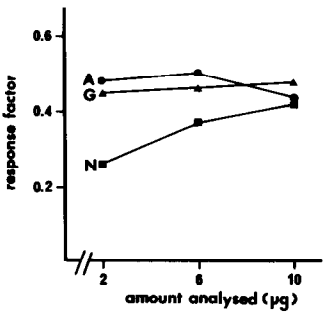

Fig. 6. Influence of amount of sample spotted on chromarods on response factor. Response factors for (●) abierixin, (■) nigericin and (▲) grisorixin were calculated as peak area/amount analysed and normalized to that of 5 μg of methyl desoxycholate.

um of our strain when the abierixin content is higher (84 mg per 100 g of dry matter (DM) mycelium). Nigericin was found to be the major antibiotic produced by the strain (527 mg per 100 g of DM mycelium). With the basal medium (without methyl oleate), the nigericin content of the mycelium was dramatically lowed (13 mg per 100 g of DM mycelium) as previously observed with the same strain grown under the same culture conditions [5]. A similar increase in antibiotic synthesis by sodium oleate was reported previously by Arima *et al.* [21] in the formation of neomycin by *Streptomyces fradiae*. In both instances, esters or soaps derived from oleic acid would stimulate the biosynthesis of branched-chain fatty acids from exogenous amino acids, which are known to be direct precursors of nigericin in *Streptomyces hygroscopicus* [22] and of neomycin in *Streptomyes fradiae* [21].

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