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IMPROVEMENT OF CHEMICAL ANALYSIS OF ANTIBIOTICS

XI*. SIMULTANEOUS FLUORODENSITOMETRIC DETERMINATION OF POLYETHER ANTIBIOTICS

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SUMMARY

A simultaneous determination of three polyether antibiotics, salinomycin, monensin and lasalocid, was established by silica gel and RP-18 high-performance thin-layer chromatography (HPTLC) using 18,19-dihydrosalinomycin and 18,19-dihydro-20-ketosalinomycin as internal standards. Fluorescent derivatives of the polyethers were prepared by reaction of their sodium salts with 1-bromoacetylpyrene and Kryptofix 222 in acetonitrile. A silica gel HPTLC plate with carbon tetrachloride–ethyl acetate–acetonitrile (50:5:10) as a solvent system and a RP-18 HPTLC plate with dichloromethane–ethyl acetate–acetone–acetonitrile (15:2:1:55) gave satisfactory separations of the five pyrenacyl esters of salinomycin, monensin, lasalocid, 18,19-dihydrosalinomycin and 18,19-dihydro-20-ketosalinomycin. Under these conditions the internal standard 18,19-dihydrosalinomycin was found to be suitable for the simultaneous determination of salinomycin and monensin, and 18,19-dihydro-20-ketosalinomycin for lasalocid. A linear relationship was obtained between the fluorescence intensity and the amount of each antibiotic in the range 2–14 ng. The detection limit of the three antibiotics was 100 pg.

INTRODUCTION

Salinomycin, monensin and lasalocid belong to a family of carboxylic polyether antibiotics (Fig. 1) and are used as feed additives in the prevention of coccidiosis in poultry[1]. Moreover, these compounds have recently been used as a growth promoter for beef cattle[2]. The increasing usage of these compounds may cause induction of resistant bacteria, residual problems in agricultural products and environmental

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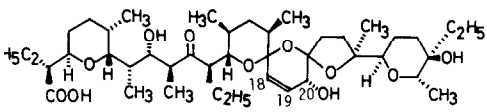
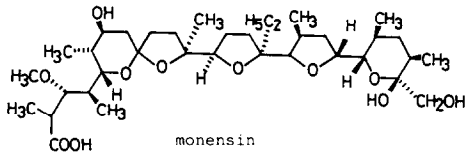


salinomycin



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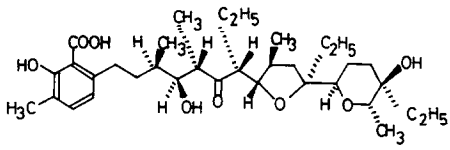

lasalocid

Fig. 1. Structures of polyether antibiotics.

pollution[3–7]. So a simple, rapid and reliable analytical method is required for their determination.

Previous assays for salinomycin and monensin have involved a colorimetric method[8], a liquid chromatographic post-column reaction system[2,9] and a colorimetric method on a thin-layer chromatographic plate[10–12]. In all these methods vanillin is used as a detection reagent. They are insensitive and unsuitable for low level residual analysis. On the other hand, lasalocid has been assayed by spectrofluorometric, high-performance liquid chromatography (HPLC)[13–19], pyrolytic gas–liquid chromatography (GLC) and pyrolytic gas–liquid chromatography–mass spectrometry[20–23]. Recently, the Analytical Methods Committee of the Royal Society of Chemistry has reported collaborative studies on residual monensin[24]. Fluorescence HPLC methods were also introduced by Martinez and Shimoda[25,26] and Takatsuki *et al.*[27] using 9-anthryldiazomethane. The colorimetric methods approved for salinomycin and monensin cannot be applied to the determination of lasalocid, which is less sensitive to vanillin[11,28]; thus the three polyethers cannot be simultaneously determined by the methods described above.

In a previous paper[29] we reported a simultaneous fluorodensitometric determination of salinomycin and monensin, but we did not apply the method to lasalocid, because at that time lasalocid was not approved in Japan for use as a feed additive in poultry. We subsequently concentrated our efforts to develop a simultaneous determination of low levels of salinomycin, monensin and lasalocid applicable to feed and residual analyses. For this purpose the problems to overcome are: (1) to improve the derivatization conditions reported previously which resulted in a low derivatization yield for lasalocid; (2) to introduce internal standards for simultaneous determination and (3) to introduce high-performance thin-layer chromatography (HPTLC) in both normal- and reversed-phase modes. Investigation of the above

points has led to the establishment of a simultaneous fluorodensitometric determination of salinomycin, monensin and lasalocid.

EXPERIMENTAL

Chemicals

Pre-coated silica gel 60 HPTLC plates (thickness 0.2 mm) and RP-18 HPTLC plates (thickness 0.2 mm) were obtained from E. Merck (Darmstadt, F.R.G.). Salinomycin sodium (SL) was supplied by Pfizer Taito (Tokyo, Japan), and monensin sodium (MN) and lasalocid sodium (LA) from Hexyst-Japan (Tokyo, Japan) and Sigma (Tokyo, Japan), respectively. 1-Bromoacetylpyrene was prepared according to the procedure of Kawahara *et al.*[30]. All other chemicals were analytical reagent grade.

Preparation of internal standards

18,19-Dihydrosalinomycin sodium (DSL). A solution of SL (6.35 g, 8.21 mmol) in 50 ml ethanol was hydrogenated in the presence of 10% palladium–carbon (612 mg) under atmospheric pressure of hydrogen for 2 days. The catalyst was filtered off and washed with ethanol. The filtrate and washings were pooled and concentrated under reduced pressure. Crude DSL (5.72 g) obtained was recrystallized from cyclohexane to give 477 mg of DSL, m.p. 166–169°C. IR, $\nu_{max.}$ in chloroform: 3350 (broad, OH), 2950, 2925, 1710 (C=O), 1550, 1450, 1400, 1375, 1110, 955 cm^{-1}. Chemical ionization mass spectrum (NH_3): *m/z* 775 (MH^+), 528 (base peak). NMR spectrum (400 MHz) in [^{2}H]chloroform: δ 0.72 (3 H, d, J = 7.1), 0.75 (3 H, t, J = 7.5), 0.78 (3 H, d, J = 6.8), 0.86 (3 H, d, J = 6.4), 0.89 (3 H, d, J = 7.6), 0.90 (3 H, t, J = 7.6), 0.93 (3 H, d, J = 7.0), 0.96 (3 H, t, J = 7.3), 1.22 (3 H, d, J = 6.8), 1.57 (3 H, s), 2.22 (1 H, dt, J = 11.3, 12.0), 2.35 (1 H, dq, J = 4.4, 12.7), 2.65–2.7 (2 H), 2.82 (1 H, dt, J = 3.4, 11.2), 3.38 (2 H, t, J = 10.7), 3.49 (1 H, dd, J = 12.4, 4.6), 3.71 (1 H, dd, J = 9.7, 1.3), 4.01 (1 H, q, J = 6.6), 4.13 (1 H, d, J = 9.7 Hz).

18,19-Dihydro-20-ketosalinomycin sodium (DKSL). A solution of SL (3.09 g, 4.00 mmol) in 100 ml of dichloromethane was added to a solution of pyridinium chlorochromate ($C_5H_5NHCrO_3Cl$, 1.67 g, 7.75 mmol) in 70 ml of dichloromethane. The mixture was stirred for 15 h then diluted in 200 ml of anhydrous diethyl ether. The ether solution was chromatographed on a Florisil column (200 × 20 mm I.D.) with diethyl ether to give crude 20-ketosalinomycin sodium (2.77 g, 3.58 mmol).

A solution of 20-ketosalinomycin sodium in 150 ml of ethanol was treated with 10% palladium–carbon (300 mg), and the solution was hydrogenated for 9 h to give DKSL (2.9 g) as a white powder, m.p. 105–108°C. IR, $\nu_{max.}$ in chloroform: 3400 (broad, OH), 2950, 2925, 1710 (C=O), 1450 cm^{-1}. Chemical ionization mass spectrum (NH_3): *m/z* 773 (MH^+), 526 (base peak). NMR spectrum (400 MHz) in [^{2}H]chloroform: δ 0.70 (3 H, d, J = 7.1), 0.79 (3 H, t, J = 7.3), 0.81 (3 H, d, J = 6.8), 0.84 (3 H, d, J = 7.3), 0.90 (3 H, t, J = 7.6), 0.93 (6 H, d, J = 6.4), 0.97 (3 H, t, J = 7.3), 1.14 (3 H, d, J = 6.6), 1.40 (3 H, s), 2.11–2.20 (2 H), 2.35 (1 H, ddd, J = 13.3, 4.9, 4.1), 2.54 (1 H, ddd, J = 12.4, 9.0, 3.4), 2.61 (1 H, dd, J = 10.7, 2.2), 2.71 (1 H, dq, J = 10.2, 7.3), 2.85 (1 H, dt, J = 3.9, 11.0), 3.10 (1 H, dt, J = 5.4, 14.0), 3.42 (1 H, dd, J = 5.6, 4.4), 3.48 (1 H, d, J = 6.3), 3.52 (1 H, d, J = 10.0), 3.58 (1 H, dd, J = 9.7, 1.7), 3.99 (1 H, dd, J = 11.0, 6.1), 4.10 (1 H, dd, J = 10.0, 5.4), 4.33 (1 H, q, J = 6.6 Hz).

Preparation of pyrenacyl esters

18,19-Dihydrosalinomycin pyrenacyl ester (DSL-PY). To a solution of DSL 200.5 mg, 0.26 mmol) in 50 ml of acetonitrile were added 1-bromoacetylpyrene (253.3 mg, 0.78 mmol) and Kryptofix 222 (109.2 mg, 0.29 mmol). The reaction mixture was stirred for 30 min and then concentrated under reduced pressure. The residue was chromatographed on a silica gel column (300 × 20 mm I.D.) with benzene–ethyl acetate (7:3) to give DSL-PY (161.5 mg) as a yellow powder, m.p. 105–110°C. IR, $\nu_{max.}$ in chloroform: 3400 (broad OH), 2950, 1690 (ester C=O, 1450, 1380, 1200 1090, 960, 840 cm^{-1}. UV, $\lambda_{max.}$ (log ε) in dioxane: 357 (4.11), 285 (4.14), 243 (4.23). Desorption chemical ionization mass spectrum (isobutane): *m/z* 995 (MH^+). NMR spectrum (400 MHz) in [^{2}H]chloroform: δ 0.58 (3 H, d, J = 7.1), 0.64 (3 H, d, J = 6.6), 0.78 (3 H, t, J = 7.6), 0.79 (3 H, d, J = 7.1), 0.89 (3 H, t, J = 6.9), 0.95 (3 H, d, J = 7.3), 0.98 (3 H, d, J = 6.8), 1.07 (3 H, d, J = 6.8), 1.20 (3 H, t, J = 7.1), 1.21 (3 H, s), 2.22 (1 H, dt, J = 11.7, 9.8), 2.56 (1 H, dt, J = 7.6, 3.8), 2.99 (1 H, dd, 10.0, 7.3), 3.01 (1 H, d, J = 4.9), 3.16 (1 H, dt, J = 3.7, 11.0), 3.27 (1 H, m), 3.38 (1 H, d, J = 11.0), 3.44 (1 H, dd, J = 10.2, 2.7), 3.68 (1 H, broad, J = 8.1), 3.66–3.72 (2 H), 4.09 (1 H, dd, J = 10.5, 4.8), 4.18 (1 H, dd, J = 11.0, 6.0), 5.55 (1 H, d, J = 17.0), 6.18 (1 H, d, J = 17.0), 8.07 (1 H, t, J = 7.6), 8.10 (1 H, d, J = 8.8), 8.20 (1 H, d, J = 9.3), 8.22 (1 H, d, J = 8.5), 8.23 (1 H, d, J = 9.5), 8.27 (2 H, dd, J = 7.8, 1.7), 8.85 (1 H, d, J = 8.0), 9.09 (1 H, d, J = 9.2 Hz).

18,19-Dihydro-20-ketosalinomycin pyrenacyl ester (DKSL-PY). To a solution of DKSL (300.3 mg, 0.39 mmol) in 50 ml of acetonitrile were added 1-bromoacetylpyrene (380.9 mg, 1.18 mmol) and Kryptofix 222 (160.2 mg, 0.43 mmol). The reaction mixture was stirred for 50 min and then concentrated under reduced pressure. The residue was chromatographed on a silica gel column (300 × 20 mm I.D.) with benzene–acetone (19:1) to give DKSL-PY (243.2 mg) as a yellow powder, m.p. 102–105°C. IR, $\nu_{max.}$ in chloroform: 3525 (broad, OH), 2925, 1720 (ester C=O), 1695, 1590, 1580, 1450, 1380, 1220, 1045, 995, 950, 840 cm^{-1}. UV, $\lambda_{max.}$ (log ε) in dioxane: 355 (4.20), 284 (4.24), 243 (4.35). D/CIMS (isobutane): *m/z* 993 (MH^+). NMR spectrum (400 MHz) in [^{2}H]chloroform: δ 0.49 (3 H, d, J = 7.2), 0.60 (3 H, d, J = 6.5), 0.78 (3 H, d, J = 6.8), 0.81 (3 H, t, J = 7.3), 0.87 (3 H, d, J = 7.0), 0.88 (3 H, t, J = 7.3), 0.97 (3 H, d, J = 6.8), 1.07 (3 H, d, J = 7.1), 1.17 (3 H, t, J = 7.1), 1.23 (3 H, s), 2.29 (1 H, m), 2.55 (1 H, ddd, J = 8.0, 4.9, 1.4), 2.68 (1 H, broad s), 2.74 (1 H, ddd, J = 16.6, 10.0, 6.3), 2.96 (1 H, dq, J = 10.0, 7.1), 3.09 (1 H, d, J = 5.4), 3.17 (1 H, dt, J = 3.9, 11.0), 3.22 (1 H, d, J = 11.5), 3.28 (1 H, dd, J = 10.5, 2.0), 3.65–3.72 (2 H), 4.03 (1 H, dd, J = 9.8, 5.7), 4.16 (1 H, dd, J = 11.0, 6.1), 5.61 (1 H, d, J = 17.0), 5.99 (1 H, d, J = 17.0), 8.06 (1 H, t, J = 7.6), 8.09 (1 H, d, J = 7.1), 8.20 (1 H, d, J = 10.0), 8.21 (1 H, d, J = 8.0), 8.22 (1 H, d, J = 9.5), 8.26 (2 H, dd, J = 7.6, 2.2), 8.73 (1 H, d, J = 8.0), 9.02 (1 H, d, J = 9.5 Hz).

Lasalocid pyrenacyl ester (LA-PY). To a solution of LA (200.1 mg, 0.33 mmol) in 50 ml of acetonitrile were added 1-bromoacetylpyrene (320.1 mg, 0.99 mmol) and Kryptofix 222 (143.1 mg, 0.36 mmol). The reaction mixture was stirred for 50 min and then concentrated under reduced pressure. The residue was chromatographed on a silica gel column (300 × 20 mm I.D.) with benzene–acetone (19:1) to give LA-PY (266.9 mg) as a yellow powder, m.p. 90–94°C. IR, $\nu_{max.}$ in chloroform: 3425 (broad, OH), 2925, 1700 (ester, C=O), 1650, 1590, 1580, 1450, 1410, 1380, 1240, 1145, 1115, 1050, 955, 845 cm^{-1}. UV, $\lambda_{max.}$ (log ε) in dioxane: 360 (4.07), 287 (4.23),

243 (4.37). Desorption chemical ionization mass spectrum (isobutane): *m/z* 834 (MH^+). NMR spectrum (400 MHz) in [^{2}H]chloroform: δ 0.73 (3 H, t, J = 7.5), 0.82 (3 H, t, J = 7.3), 0.83 (3 H, t, J = 7.3), 0.94 (3 H, d, J = 7.1), 0.95 (3 H, d, J = 6.6), 0.97 (3 H, d, J = 6.6), 1.11 (3 H, d, J = 6.8), 2.15 (1 H, m), 2.26 (3 H, s), 2.74 (1 H, dt, J = 10.3, 3.4), 2.89–2.98 (2 H, m), 3.12 (1 H, dt, J = 4.9, 10.7), 3.25 (1 H, br), 3.39 (1 H, dd, J = 11.7, 2.2), 3.73 (1 H, q, J = 6.8), 3.81 (1 H, dd, J = 10.0, 3.9), 4.05 (1 H, d, J = 9.0), 6.73 (1 H, d, J = 7.5), 7.20 (1 H, d, J = 7.5), 8.09 (1 H, t, J = 7.6), 8.11 (1 H, d, J = 9.0), 8.22 (1 H, d, J = 8.8), 8.23 (1 H, d, J = 8.3), 8.27 (1 H, d, J = 9.3), 8.29 (2 H, dd, J = 7.6, 3.7), 8.47 (1 H, d, J = 8.1), 9.13 (1 H, d, J = 9.3 Hz).

Solvent systems

For silica gel HPTLC, carbon tetrachloride–ethyl acetate–acetonitrile (50:5:10) was used, and for RP-18 HPTLC, dichloromethane–ethyl acetate–acetone–acetonitrile (15:2:1:55).

Thin-layer chromatography

Each 0.5-μl solution was applied on silica gel HPTLC and RP-18 HPTLC plates with a microsyringe and the plates were developed by the ascending technique until the front reached a height of 10 cm using the solvent systems described above. Detection of spots was carried out by exposure to a UV lamp (365 nm).

Densitometry

The developed HPTLC plates were examined by a Shimadzu CS-910 dual-wavelength chromatoscanner (Shimadzu, Kyoto, Japan) and the spots were determined fluorodensitometrically. Conditions: fluorescence mode; wavelength, $\lambda_{excitation}$ = 360 nm, $\lambda_{emission}$ = 450 nm (interference filter); linear scanning in reflection mode; size of scanning beam, 9.0 mm × 1.25 mm.

Simultaneous determination

Amounts of 20 mg of SL, MN and LA were accurately weighed into a 50-ml volumetric flask and diluted to volume in acetonitrile. A 10-ml volume of the resulting solution was pipetted into a 50-ml volumetric flask, diluted to volume in the same solvent; 1, 2, 4, 6 and 7 ml of this solution were pipetted into 20-ml volumetric flasks.

Amounts of 20 mg of DSL and DKSL were accurately weighed into a 50-ml volumetric flask and diluted to volume in acetonitrile. A 10-ml volume of this solution was pipetted into a 25-ml volumetric flask and diluted to volume in acetonitrile; 2 ml of this solution were pipetted into the five 20-ml volumetric flasks containing the solutions of SL, MN and LA added previously. Furthermore, 150 mg of 1-bromoacetylpyrene and 40 mg of Kryptofix 222 were each weighed into 50-ml volumetric flasks and diluted to volume in acetonitrile; 5 ml of these solutions were pipetted into the five 20-ml volumetric flasks containing the samples (SL, MN and LA) and the internal standards (DSL and DKSL), and diluted in acetonitrile to 20 ml. The final concentrations of the samples (SL, MN and LA) in the five volumetric flasks were 4, 8, 16, 24 and 28 μg/ml, respectively, and those of the internal standards DSL and DKSL were 16 μg/ml. The volumetric flasks were allowed to stand in an oil-bath at 50°C for 90 min. After cooling to room temperature, 0.5-μl quantities of these so-

lutions were applied using a microsyringe on silica gel and RP-18 HPTLC plates. After developing with the solvent described above, the spots were determined by a fluorodensitometer.

RESULTS AND DISCUSSION

In the case of the simultaneous fluorodensitometric determination of SL, MN and LA directed toward residual analysis, the extraction, clean-up, derivatization and separation steps must be reproducible. Therefore, we introduced internal standards to establish a reliable method. Secondly, LA was found to have lower reactivity for pyrenacylation than SL and MN in a preliminary experiment; thus we reinvestigated the derivatization conditions for simultaneous determination of the three polyethers. Thirdly, a reliable separation system for five pyrenacyl esters (SL, MN, LA and two internal standards) was sought using silica gel and RP-18 high-performance thin-layer chromatography (HPTLC) plates. In order to establish a simultaneous analytical method for the three polyether antibiotics using internal standards, we examined the parameters of the analysis in detail and the results are discussed below.

Internal standards

It is difficult to obtain a constant derivatization yield in feed and residual analyses because this might vary with the amount of coexisting materials. The best way to eliminate such problems is to introduce an internal standard in the analytical system. In the present method the internal standard must have a similar reactivity for pyrenacylation, a similar R_F value on a TLC plate to those of SL, MN and LA and must not be contained in a sample as an impurity. A compound which fulfils these conditions is a structural analogue of the three antibiotics. Thus, SL derivatives, 18,19-dihydrosalinomycin (DSL) and 18,19-dihydro-20-ketosalinomycin (DKSL), were expected to be effective as internal standards. DSL was obtained by hydrogenation of SL in the presence of 10% palladium–carbon and DKSL was prepared by oxidation of SL with pyridinium chlorochromate followed by catalytic hydrogenation.

Separation

In a previous paper we reported that Kryptofix 222 was very effective in the transformation of a carboxylate group to the corresponding pyrenacyl ester[29]. Using

TABLE I

EXCITATION AND EMISSION MAXIMA OF PYRENACYL ESTERS

Pyrenacyl ester derivative	*Excitation maximum (nm)*	*Emission maximum (nm)*
Salinomycin	361	406
18,19-Dihydrosalinomycin	359	408
18,19-Dihydro-20-ketosalinomycin	355	410
Monensin	352	409
Lasalocid	357	426

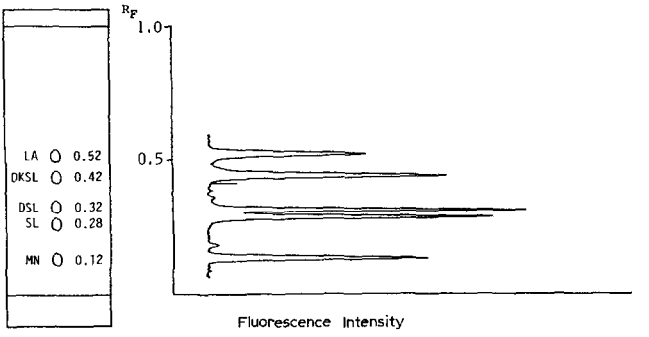

Fig. 2. Separation of pyrenacyl esters on silica gel (E. Merck, 5641) HPTLC and the densitometric profile. Solvent system: carbon tetrachloride–ethyl acetate–acetonitrile (50:5:10). SL, DSL, DKSL, MN and LA are chromatographed as their pyrenacyl esters.

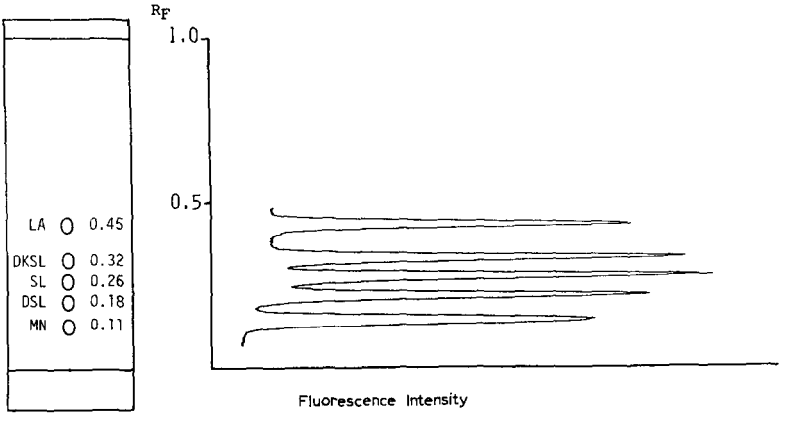

Fig. 3. Separation of pyrenacyl esters on RP-18 (E. Merck, 5914) HPTLC and the densitometric profile. Solvent system: dichloromethane–ethyl acetate–acetone–acetonitrile (15:2:1:55). SL, DSL, DKSL, MN and LA are chromatographed as their pyrenacyl esters.

this catalyst we synthesized the authentic pyrenacyl esters of SL, MN, LA, DSL and DKSL, and their structures were confirmed by nuclear magnetic resonance and infrared spectroscopy and mass spectrometry. The fluorescence spectral data for these compounds are summarized in Table I.

We studied the separation conditions of these pyrenacyl esters. Although, in the case of the simultaneous determination of SL and MN, hexane–ethyl acetate (2:3) showed a good separation on a silica gel TLC plate[29], this solvent system was found to be unsatisfactory in the present case. The spots of DSL-PY and DKSL-PY overlapped with those of salinomycin pyrenacyl ester (SL-PY) and LA-PY, respectively. The separations and the densitometric profiles of the pyrenacyl esters on silica gel and RP-18 HPTLC plates obtained under optimized conditions are illustrated in Figs. 2 and 3, respectively. A satisfactory result was obtained on a silica gel HPTLC plate using carbon tetrachloride–ethyl acetate–acetonitrile (50:5:10). Acetonitrile in the system brought about a satisfactory and reproducible separation between SL-PY and DSL-PY. In the case of RP-18 HPTLC, dichloromethane–ethyl acetate–

acetone–acetonitrile (15:2:1:55) yielded good resolution of five pyrenacyl esters; the small amount of acetone was used to obtain distinct spots.

Derivatization

We examined the derivatization conditions (molar ratio, reaction temperature and reaction time) for DSL, DKSL and LA at a concentration of 20 μg/ml. Reaction yields were calculated from standard curves for authentic pyrenacyl esters determined by fluorodensitometry on a silica gel HPTLC plate after development. As reported in a prevous paper[29], SL was successfully pyrenacylated at a 1:3:2 molar ratio of SL to 1-bromoacetylpyrene to Kryptofix 222 and the reaction temperature was 25°C. So, we adopted this condition to derivatize DSL and DKSL. The reaction yields of DSL were 67.3% (reaction time, 30 min), 88.5% (60 min), and 90.4% (90 min), and those of DKSL were 52.5% (30 min), 77.3% (60 min) and 81.1% (90 min). Although the derivatization yields of DSL and DKSL were slightly low compared with those of SL, *i.e.*, 84.2% (30 min), 98.4% (60 min) and 94.6% (90 min), they are reproducible and tolerable for the present analysis. Next, the reaction of LA with 1-bromoacetylpyrene was attempted under the same conditions as for SL but in contrast to the case of SL the reaction did not proceed. So we used the previously reported conditions for MN (molar ratio 1:20:10, reaction temperature 50°C, reaction time 90 min)[29] but the reaction yield of LA was only 36.8%. When the amount of 1-bromoacetylpyrene was increased to a 50-fold molar excess, the reaction yield rose to 80.4%. Increasing of the amount of the catalyst did not significantly affect the reaction yield in the presence of this amount of 1-bromoacetylpyrene. The molar ratio finally chosen was 1:50:10. The optimum derivatization conditions for producing pyrenacyl esters are summarized in Table II.

In these experiments, it was revealed that the relative reactivity of the polyethers lies in the following order; SL > DSL ≈ DKSL > MN > LA. The low reactivity of LA was considered to be due to the presence of an aromatic carboxylate with a hydroxy group in an *ortho* position. So, we examined the pyrenacylation of two model compounds (20 μg/ml), namely sodium salicylate as an *o*-hydroxybenzoic acid derivative and sodium benzoate as an aromatic carboxylic acid. Under the derivatization conditions used for SL, a strong fluorescent spot appeared immediately on a TLC plate in the case of sodium benzoate, whereas in the case of sodium salicylate

TABLE II

OPTIMUM DERIVATIZATION CONDITIONS FOR SALINOMYCIN (SL), 18,19-DIHYDRO-SALINOMYCIN (DSL), 18,19-DIHYDRO-20-KETOSALINOMYCIN (DKSL), MONENSIN (MN) AND LASALOCID (LA)

Compound	*Molar excess*		*Reaction temperature (°C)*	*Reaction time (min)*
	Reagent	*Catalyst*		
SL	3-fold	2-fold	25	90
DSL	3-fold	2-fold	25	90
DKSL	3-fold	2-fold	25	90
MN	20-fold	10-fold	50	90
LA	50-fold	10-fold	50	90

TABLE III

CHARACTERISTICS OF STANDARD CURVES ON SILICA GEL AND RP-18 HPTLC PLATES USING THE INTERNAL STANDARDS, 18,19-DIHYDROSALINOMYCIN (DSL) AND 18,19-DIHYDRO-20-KETOSALINOMYCIN (DKSL)

*Sample**	*Internal standard**	*Silica gel HPTLC*		*RP-18 HPTLC*	
		Regression equation	*Correlation coefficient*	*Regression equation*	*Correlation coefficient*
Salinomycin (SL)	DSL	$y = 0.060x + 0.019$	0.9999	$y = 0.074x - 0.039$	0.9999
	DKSL	$y = 0.087x + 0.031$	0.9993	$y = 0.064x - 0.032$	0.9994
Monensin (MN)	DSL	$y = 0.058x - 0.022$	0.9999	$y = 0.077x + 0.038$	0.9999
	DKSL	$y = 0.078x + 0.222$	0.9993	$y = 0.060x + 0.107$	0.9985
Lasalocid (LA)	DSL	$y = 0.032x - 0.056$	0.9995	$y = 0.065x - 0.048$	0.9985
	DKSL	$y = 0.044x - 0.062$	0.9997	$y = 0.056x - 0.027$	0.9996

* These were determined as pyrenacyl esters.

no fluorescence was detected after 30 min and a weak fluorescent spot appeared after 60 min. These experiments showed that the low reactivity of LA to pyrenacylation is due to the *o*-hydroxybenzoic acid moiety.

Simultaneous determination of salinomycin, monensin and lasalocid

Before starting the simultaneous determination of SL, MN and LA, we examined the determination of the individual samples listed in Table II using the corresponding derivatization conditions. Calibration plots were linear between 2 and 14 ng for each sample on both silica gel and RP-18. Therefore, we attempted the simultaneous determination of SL, MN and LA with a constant concentration (16 μg/ml) of DSL and DKSL which are expected to be effective as internal standards. As described in Experimental, the following derivatization conditions were used: molar ratio 1:50:10, reaction temperature 50°C, reaction time 90 min, as used for LA. After derivatization, 0.5 μl of the reaction mixture were spotted on silica gel and RP-18 HPTLC plates and developed with the solvent systems. The intensity of the fluorescence was recorded by a fluorodensitometer under the conditions described under Experimental.

First, we describe the results on silica gel. Good separation of the five pyrenacyl esters from 1-bromoacetylpyrene (R_F 0.76) was obtained. Five fluorescent peaks of the pyrenacyl esters appeared distinctly in the densitometric profiles. We examined whether DSL and DKSL are effective as internal standards in the determination of the polyether antibiotics. Calculation of the peak height ratios of SL-PY/DSL-PY, monensin pyrenacylester (MN-PY)/DSL-PY and LA-PY/DSL-PY, and also of SL-PY/DKSL-PY, MN-PY/DKSL-PY and LA-PY/DKSL-PY, gave calibration curves of which the regression equations and correlation coefficients were evaluated. The data shown in Table III indicate a good correlation for each combination and that both DSL and DKSL functioned as good internal standards. Considering the densitometric profile (Fig. 2), we recommend the following pairs (sample/internal standard), SL/DSL, MN/DSL and LA/DKSL, which showed higher correlation coefficients (Table III). The two internal standards, however, can be used complementarily depending on the nature of the actual sample where many interfering substances may exist.

On RP-18, five fluorescent peaks appeared with good resolution as illustrated in Fig. 3. The peaks of 1-bromoacetylpyrene (R_F 0.80) and its decomposition products (R_F 0.66 and 0.53) did not interfere with the measurement of the five pyrenacyl esters. As in the case of silica gel, calibration curves were obtained for six pairs of sample and internal standard, SL-PY/DSL-PY, MN-PY/DSL-PY, LA-PY/DSL-PY, SL-PY/DKSL-PY, MN-PY/DKSL-PY and LA-PY/DKSL-PY and their regression equations and correlation coefficients were calculated. As shown in Fig. 3 and Table III, the internal standard DSL was effective for the determination of SL and MN, and DKSL for LA.

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