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IMPROVEMENT OF CHEMICAL ANALYSIS OF ANTIBIOTICS

IV*. FLUORODENSITOMETRIC DETERMINATION OF POLYETHER ANTIBIOTICS

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SUMMARY

A fluorodensitometric determination of the polyether antibiotics, salinomycin and monensin, on silica gel thin-layer plates based on fluorescence labelling of carboxylate with 1-bromoacetylpyrene has been established. The optimum conditions for producing salinomycin 1-pyrenacyl ester are described. Using Kryptofix 222 as a catalyst, the reaction fluorescence yield is significantly higher than using dicyclohexyl-18-crown-6. There is a linear relationship between the fluorescence intensity and the amount of salinomycin and monensin between 2 and 14 ng and the detection limits are 100 pg.

INTRODUCTION

Salinomycin, monensin and lasalocid are polyether antibiotics (Fig. 1) produced by *Streptomyces* which have a microbial activity against gram-positive bacteria, mycobacteria, fungi and a number of viruses. Furthermore, these compounds are also effective in the treatment of coccidial infection in poultry. They are used at about 60–125 ppm in broiler feed in many countries[1].

Microbiological assays for these compounds have been achieved with turbidimetric[2,3], cylinder plate[4–7] and thin-layer bioautographic methods[8]. Since the microbiological assays are time-consuming and uncertain, a rapid and reproducible chemical assay is required. Because lasalocid has a unique cresotic acid moiety which causes a significant fluorescence in various organic solvents, a spectrofluorometric method has been established[9–15]. Pyrolytic gas–liquid chromatographic (GLC)[16–18] and pyrolytic gas–liquid chromatographic–mass spectrometric (GC–MS) methods[19] have also been reported. However, salinomycin and monensin have no chromophore

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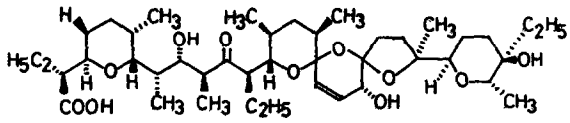
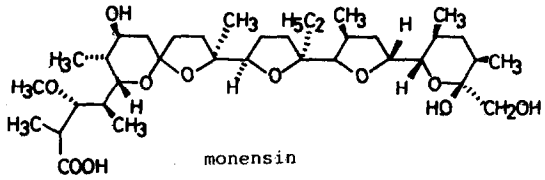
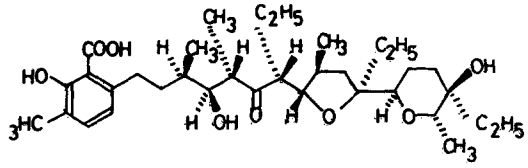


salinomycin



monensin



lasalocid

Fig. 1. Structures of polyether antibiotics.

and GLC is hard to apply due to their involatility and instability. At present, only a colorimetric method is frequently used for the analysis[20]. In this method a solution of 3% vaniline in 0.5% sulphuric acid in methanol is used as a colour-producing reagent and the method is adaptable to feed premix, fermentation broth and crystalline samples. However, since this method is insensitive and interference from other materials cannot always be excluded, it is not suitable for residue analysis. Although Macy and Loh[21] established a high-performance liquid chromatographic (HPLC) determination of monensin in feed premix with a refractive index detector, this method has poor sensitivity for feed and residue analysis.

Recently, we established a simple method for the analysis of tetracyclines (TCs) with a precoated silica gel HPTLC plate[22] and reversed-phase thin-layer chromatographic (TLC) plate[23] followed by densitometry, and successfully applied it to the analysis of TCs in fish tissues[24]. Therefore, we attempted to apply a TLC–fluorodensitometric technique for the development of a faster, more specific and more sensitive assay of salinomycin and monensin. This paper describes its optimization for fluorometric detection and quantitative analysis of these compounds.

EXPERIMENTAL

Chemicals

Pre-coated silica gel 60 TLC plates (thickness 0.25 mm) were purchased from E. Merck (Darmstadt, F.R.G.). Salinomycin was supplied by Pfizer Taito Co. (Tokyo, Japan) and monensin was purchased from Hexyst-Japan Co. (Tokyo, Japan). 1-Bromoacetylpyrene was prepared to according to the procedure of Kawahara *et al.*[25]. All other chemicals were analytical reagent grade.

Preparation of salinomycin 1-pyrenacyl ester

To a solution of salinomycin sodium (500.6 mg, 0.648 mmol) in acetonitrile was added 1-bromoacetylpyrene (627.7 mg, 1.943 mmol) and dicyclohexyl-18-crown-6 (482.3 mg, 1.293 mmol). The solution was stirred for 150 min and then concentrated under reduced pressure. The residue was chromatographed on a silica gel column (350 × 25 mm I.D.; No. 7734, E. Merck, Elmsford, NY, U.S.A.) with benzene–ethyl acetate (3:1). A crude salinomycin 1-pyrenacyl ester (601.9 mg) was obtained and recrystallized from benzene–cyclohexane to give 342.8 mg of salinomycin 1-pyrenacyl ester as a colourless needles, m.p. 138–140°C. Calc. for $C_{60}H_{80}O_{12}$: C, 72.55; H, 8.11%. Found: C, 72.22; H, 8.11%. IR, ν_{max} in chloroform: 1730, 1700 cm^{-1} (C=O). NMR in deuterochloroform: δ 9.18 (d, 1H, J = 9 Hz), 8.91 (d, 1H, J = 8 Hz), 8.32–7.86 (m, 7H), 6.88 (d, 1H, J = 18 Hz), 5.96 (s, 2H), 5.60 ppm (d, 1H, J = 18 Hz). UV, λ_{max} (log ε) in dioxane: 235 (4.48), 243 (4.49), 286 (4.32), 358 (4.28), 392 nm (3.97). $[\alpha]_D$: −24.4° (c = 0.35, $CHCl_3$).

Preparation of monensin 1-pyrenacyl ester

To a solution of monensin sodium (299.7 mg, 0.432 mmol) in acetonitrile was added 1-bromoacetylpyrene (416.6 mg, 1.290 mmol) and dicyclohexyl-18-crown-6 (342.1 mg, 0.871 mmol). The solution was stirred for 150 min and then concentrated under reduced pressure. The residue was chromatographed on a silica gel column with benzene–ethyl acetate (3:1) and then on a preparative silica gel thin-layer plate. A monensin 1-pyrenacyl ester (120.5 mg) was obtained as a yellowish powder, m.p. 65–70°C. Calc. for $C_{54}H_{70}O_{12}$: C, 71.03; H, 7.95%. Found: C, 70.88; H, 8.32%. IR, ν_{max} in chloroform: 1735 cm^{-1} (C=O). NMR in deuterochloroform: δ 8.85 (d, 1H, J = 9 Hz), 8.36 (m, 8H), 5.52 ppm (s, 2H). UV, λ_{max} (log ε) in dioxane: 235 (4.46), 242 (4.49), 283 (4.24), 390 nm (3.81). $[\alpha]_D$: +55.3° (c = 0.52, $CHCl_3$).

Salinomycin and monensin 1-pyrenacyl ester standard solution

Each 100-mg amount of salinomycin and monensin 1-pyrenacyl ester was dissolved in 20-ml of ethyl acetate. This stock solution should be stored at 4°C and the standard solution was prepared freshly each day by diluting in ethyl acetate to a final concentration of 2–35 μg/ml.

Thin-layer chromatography

Each standard solution was applied on the silica gel TLC plate with a microsyringe and the plate was developed by the ascending technique until the front had reached a height of 10 cm using appropriate solvent systems. In order to monitor the esterification of each compound, the solvent systems used were benzene–ethyl acetate (3:2) for salinomycin 1-pyrenacyl ester and benzene–ethyl acetate (1:1) for monensin 1-pyrenacyl ester; the R_F values were 0.36 and 0.45, respectively. For simultaneous determination of these compounds, the solvent system examined was *n*-hexane–ethyl acetate (2:3); the R_F values were 0.50 and 0.35 for salinomycin 1-pyrenacyl ester and monensin 1-pyrenacyl ester, respectively.

Detection of spots

After air drying, the spots were detected by exposure to a UV lamp (365 nm).

Densitometry

The developed TLC plate was examined by a Shimadzu CS-910 dual wavelength chromatoscanner (Shimadzu, Kyoto, Japan) and the spots of the components were determined fluorodensitometrically. Operating conditions: fluorescence mode; wavelengths, $\lambda_{excitation}$ = 360 nm, $\lambda_{emission}$ = 450 nm (interference filter); linear scanning in reflection mode; size of scanning beam, 0.25 × 9.0 mm.

RESULTS AND DISCUSSION

Because salinomycin and monensin are produced as sodium salts, we undertook the fluorescence derivatization of the carboxylate group in these compounds. Although 4-bromomethyl-7-methoxycoumarin[26] and 1-bromoacetylpyrene[27] are both considered to be suitable for this purpose, we selected 1-bromoacetylpyrene because of its higher detection limit, higher reactivity and longer exitation wavelength (360 nm)[28]. Kawahara *et al.*[27] reported the synthesis and reactivity of 1-bromoacetylpyrene as a fluorescence label for carboxylic acids, and Tsuji and co-workers[29–32] investigated the HPLC of bile acids and their conjugates using this reagent. The compounds were generally labelled in acetonitrile using dicyclohexyl-18-crown-6 as a catalyst. At first, we applied this derivatization procedure for salinomycin but found that the reaction was incomplete. So we examined various reaction conditions such as catalyst, molar ratio, reaction temperature and reaction time.

Standard curve

In order to calculate the reaction yield, we synthesized each pyrenacyl ester and various amounts were spotted on a TLC plate. After developing, the fluorescence of each spot was recorded by a densitometer. The calibration graphs for salinomycin and monensin 1-pyrenacyl esters were straight lines for amounts between 1.0 and 17.5 ng. As the result of this experiment, the reaction yield could be calculated from these standard curves.

Choice of catalyst

Durst *et al.*[33,34] reported that crown ether caused a great increase in the formation of phenacyl esters. Tsuji and co-workers[29–32] reported that free and glycine-conjugated bile acids were labelled with 1-bromoacetylpyrene in acetonitrile using dicyclohexyl-18-crown-6. At first, in our derivatization study, we investigated the reactivity of salinomycin with 1-bromoacetylpyrene using dicyclohexyl-18-crown-6 under the conditions described in Fig. 2. However, the reaction yield was very low and with other crown ethers the reaction yields were less than 10% as is seen in Fig. 2A. Therefore, we examined several Kryptofixes (Kryptofix 5, 21, 22 and 222). Among them, Kryptofix 222 effectively converted salinomycin into its 1-pyrenacyl ester. The capability of the catalyst was clearly recognized even under the lower level conditions (salinomycin, 20 nmol/ml) shown in Fig. 2B. That is, while the yield was less than 10% using dicyclohexyl-18-crown-6, Kryptofix 222 catalyzed the reaction and resulted in a high yield. Consequently, we used Kryptofix 222 as a catalyst instead of dicyclohexyl-18-crown-6 in this derivatization study.

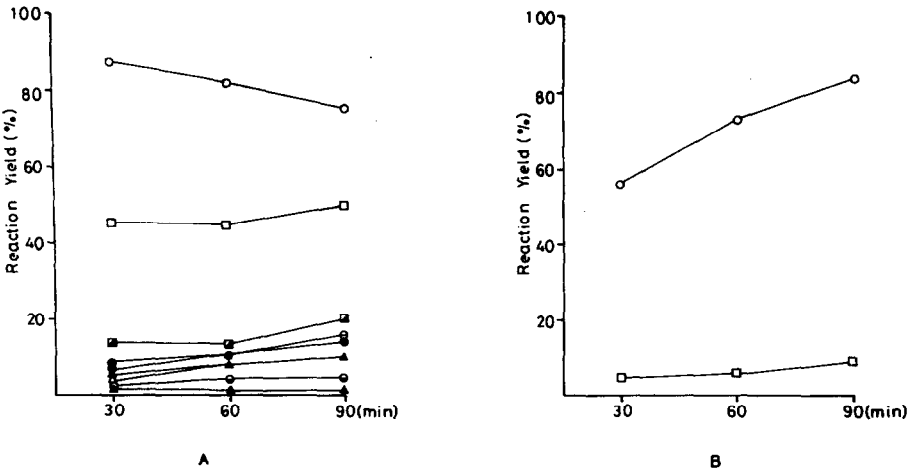


Fig. 2. Effect of the catalyst on the reactivity of salinomycin with 1-bromoacetylpyrene. Catalysts: ▲, 12-crown-4; ◪, 15-crown-5; □, dicyclohexyl-18-crown-6; ▲, dibenzo-24-crown-8; △, dibenzo-27-crown-9; ⊖; Kryptofix 5; ◒, Kryptofix 21; ●, Kryptofix 22; ○, Kryptofix 222. Salinomycin was dissolved in acetonitrile (A, 1 μmol/ml; B, 20 nmol/ml). To 4 ml of this solution, 4 ml of 1-bromoacetylpyrene and 4 ml of catalyst in acetonitrile solutions were added to give a total volume of 25 ml (molar ratio of salinomycin: 1-bromoacetylpyrene: catalyst = 1:3:2). After standing at room temperature, each fluorescence intensity was measured under the conditions described in Experimental.

Effect of molar ratio

1-Bromoacetylpyrene has been used in about 50–200 fold molar excess with dicyclohexyl-18-crown-6 as a catalyst in the derivatization of bile acids and carboxylic acids[29–32]. The relationship between the reaction yield and molar ratio (salinomycin: 1-bromoacetylpyrene: Kryptofix 222) was examined (Fig. 3A). First, the three compounds were allowed to react in a 1:1:1 molar ratio in acetonitrile at 25°C and the reaction yield was only 50%. In the case of 1:1:2 molar ratio the reaction yield was not increased. With a molar ratio of 1:3:1, the reaction yield increased to >70%. When a more than three-fold excess of 1-bromoacetylpyrene was employed the reaction yield did not increase significantly. Because the molar ratio of the catalyst did not significantly affect the reaction yield in the presence of this molar ratio of 1-bromoacetylpyrene, the molar ratio finally chosen was 1:3:2.

Effect of reaction temperature

In the cases of bile acids and carboxylic acids, the reaction temperature employed was 40–80°C using dicyclohexyl-18-crown-6[29–32]. The influence of reaction temperature was examined as shown in Fig. 3B. The reaction temperatures employed were 25, 40, 60 and 80°C, and the reaction yield was monitored during 30–90 min. Although the reaction yields were maximal at elevated temperatures, the reaction proceeded readily even at 25°C (room temperature). For convenience, the reaction temperature chosen was 25°C.

Effect of reaction time

The optimum reaction time was determined (Fig. 3C) at the molar ratio of

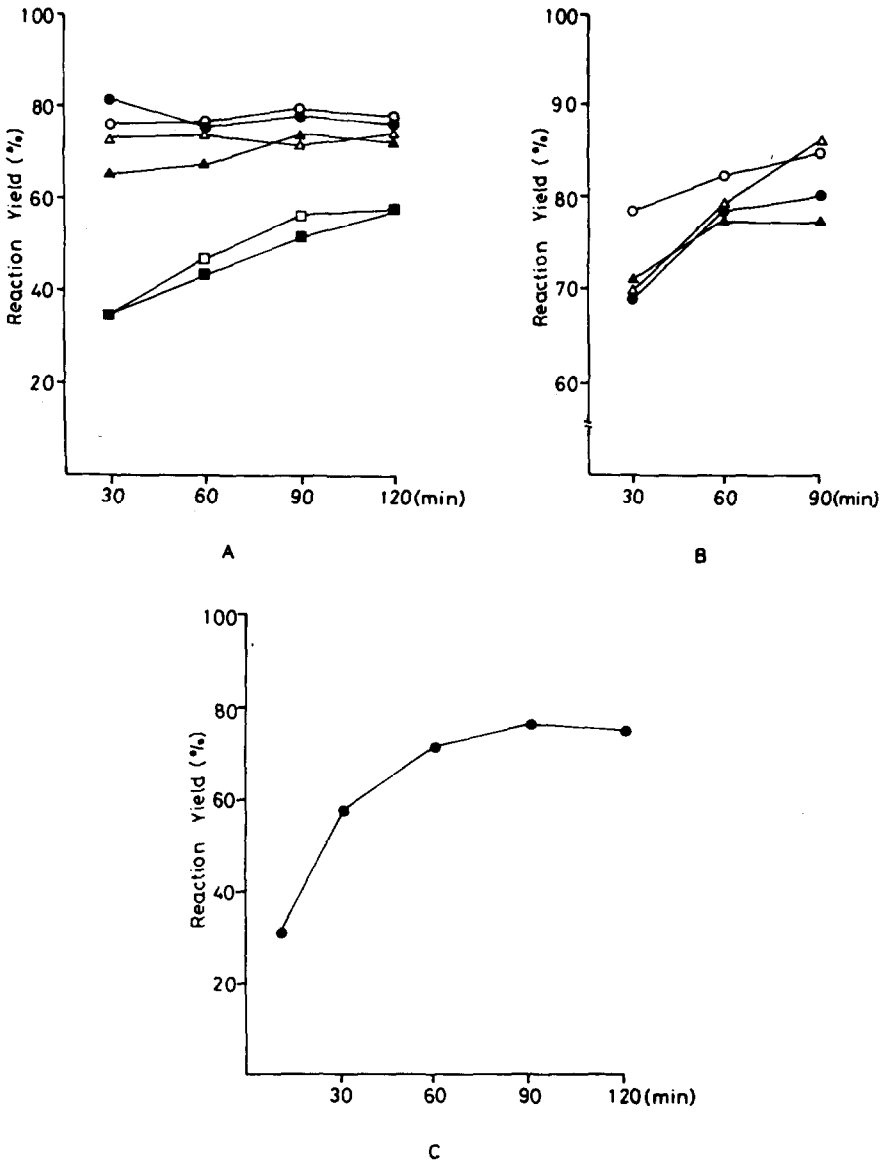


Fig. 3. Effects of the molar ratio (A), reaction temperature (B) and reaction time (C) on the reactivity of salinomycin with 1-bromoacetylpyrene. Molar ratios (A): ○, (1:3:3); ●, (1:3:2); △, (1:3:1); ▲, (1:2:2); □, (1:1:2); ■, (1:1:1). Temperatures (B): ●, 25; ○, 40; ▲, 60; △, 80°C.

1:3:2 and a reaction temperature of 25°C (room temperature). The reaction yield was reached a maximum of about 90 min and then decreased. As the result the reaction time was chosen as 90 min.

Calibration curve of salinomycin

The optimum conditions for producing salinomycin 1-pyrenacyl ester are

TABLE I

DERIVATIZATION CONDITIONS

Compound	*Molar excess*		*Reaction temperature (°C)*	*Reaction time (min)*
	Reagent	*Catalyst*		
Salinomycin	3-fold	2-fold	25	90
Monensin	20-fold	10-fold	50	90

summarized in Table I. We applied these conditions to obtain the calibration curve. Fig. 4A shows a typical result with a linear relationship between 2.0 and 14.0 ng, and the detection limit was 100 pg. Those results demonstrate that the derivatization proceeds quantitatively at 25°C (room temperature) using Kryptofix 222 as a catalyst.

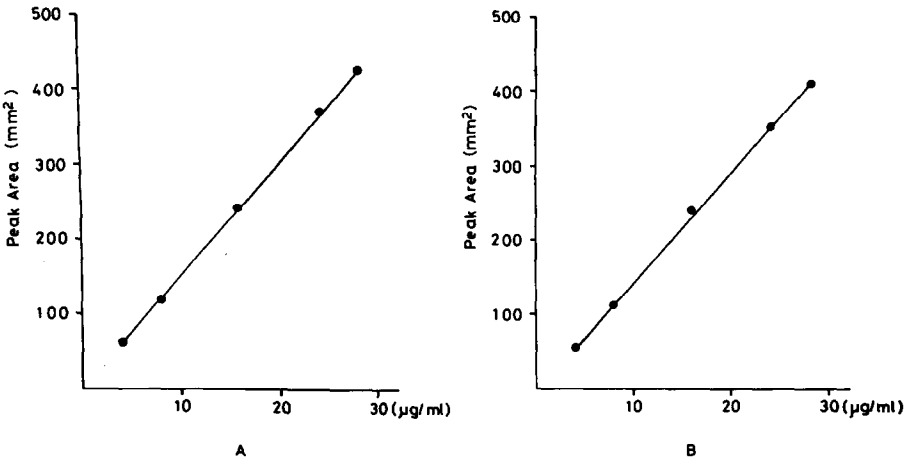


Fig. 4. Calibration curves for salinomycin (A) and monensin (B). For salinomycin: $y = 15.4x + 0.3$; $r = 0.9999$. For monensin: $y = 14.8x - 1.8$; $r = 0.9996$.

Calibration curve of monensin

In the same manner, the reaction of monensin with 1-bromoacetylpyrene was examined under the optimum conditions for salinomycin. However, in contrast to the case of salinomycin, the reaction yield was only 10–15%. When the molar ratio of 1-bromoacetylpyrene to Kryptofix 222 to monensin was 20:10:1 and the reaction temperature was 50°C, the reaction yield gradually increased from 50 to 90%. The optimum conditions for producing monensin 1-pyrenacyl ester are summarized in Table I. Subsequently, we applied these conditions for the reaction of monensin with 1-bromoacetylpyrene. Fig. 4B shows a typical result, a linear relationship between 2.0 and 14.0 ng with a good coefficient variation and with a detection limit of 100 pg. The difference in reactivity between salinomycin and monensin is under investigation.

Simultaneous determination of salinomycin and monensin

Simultaneous determination of salinomycin and monensin in biological samples, foods, premix and feed is required. Because both solvent systems used to monitor the esterification of salinomycin and monensin did not result in a good separation between the compounds, various solvent systems were tested. Among them *n*-hexane–ethyl acetate (2:3) was found to yield a good separation. Next we examined the reaction conditions and found that the pyrenacyl ester of salinomycin was produced with good reproducibility under the same conditions as in the case of monensin, without decomposition. Therefore, we achieved simultaneous determination of salinomycin and monensin under the conditions mentioned above. A typical fluorodensitogram is shown in Fig. 5, and the calibration curves for salinomycin and monensin were parallel to each other with good linearity and reproducibility.

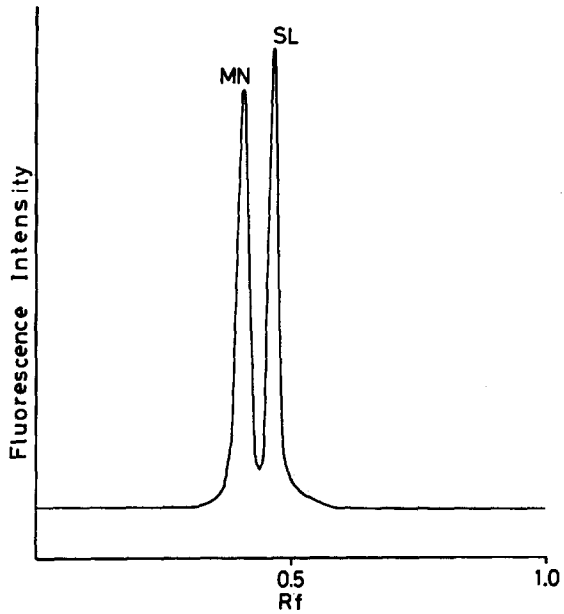


Fig. 5. Typical densitometric profiles for salinomycin (SL) and monensin (MN).

At present, we are attempting to develop a clean-up system using a pre-packed cartridge[24] for salinomycin and monensin in feed and biological samples. The results of the combination of fluorodensitometry and the clean-up system will be reported elsewhere.

In conclusion, a technique for the determination of salinomycin and monensin using fluorodensitometry has been established.

This may be a more reliable method than conventional microbiological and colorimetric assays. The fluorodensitometric technique is rapid, reproducible and highly sensitive.

Kryptofix 222 is very effective in the transformation of a carboxylate group into its pyrenacyl ester and we are investigating its catalytic effect on other classes of compounds.

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