TETRANACTIN, A NEW MITICIDAL ANTIBIOTIC
V. QUANTITATIVE DETERMINATION OF MACROTETROLIDE ANTIBIOTICS

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A method for quantitative determination of tetranactin and other macrotetrolide antibiotics is presented. The assay is based on the complex formation of the antibiotics with sodium picrate followed by extraction of the complexes into an organic solvent. The macrotetrolide antibiotics can be determined by the present method with accuracy and simplicity in the assay range of 1~40 μg/ml.

In our screening for pesticidal antibiotics a new macrotetrolide antibiotic, tetranactin, was isolated in crystalline form accompanied by two other macrotetrolide antibiotics[1,2]. Tetranactin shows remarkable and selective miticidal activity both in laboratory and field tests[3,4]. It was necessary to develop an assay method for quantitative determination of the antibiotic. However, bioassay using mites and microorganisms is troublesome and inaccurate. Macrotetrolide antibiotics, such as nonactin, monactin and dinactin[5,6], are known to form complexes with alkali metal cations and to exhibit a high degree of cation specificity in metabolic behavior[7,8]. X-Ray crystallographic studies on nonactin potassium thiocyanate complex indicated that the conformation resembles the seam of a tennis ball with K^+ ion at the center of the ball with the methyl and methylene groups of the tetrahydrofurane rings on the outside[9]. This was supported by NMR studies which showed that the macrotetrolide antibiotics are present as complexes in either acetone or aqueous acetone solutions containing alkali metal cations[10].

In this paper we report a new assay methood for the quantitative determination of macrotetrolide antibiotics which is based on their complex forming properties with sodium picrate.

Materials and Methods

Tetranactin, $C_{44}H_{72}O_{12}$, used in this study was produced by a pilot plant fermentor at Chugai Research Laboratories. Nonactin, $C_{40}H_{64}O_{12}$, dinactin, $C_{42}H_{68}O_{12}$, and trinactin, $C_{43}H_{70}O_{12}$, were generous gifts from Prof. W. KELLER-SCHIERLEIN of the Zürich Institute of Technology. Sodium picrate, sodium hydroxide, methanol and dichloromethane were analytical grade reagents of the highest purity. Palmitic and oleic acids, tripalmitin, triolein and cholesterol were obtained from Tokyo Kasei Co., Ltd. Phosphatidylcholine is a product of Nishin Seiyu Co., Ltd. These chemicals were used without further purification.

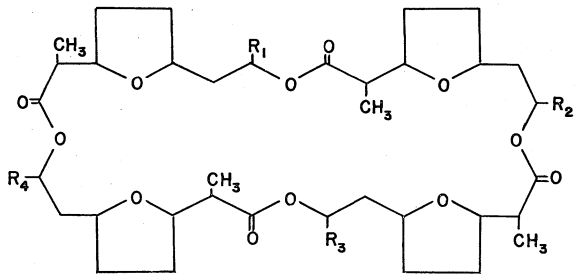
Assay Procedure: Sodium picrate, $C_6H_2(NO_2)_3ONa \cdot H_2O$ (400 mg), was dissolved in

distilled water and then sodium hydroxide (4 g) was added. The mixture was made up to 1 liter with distilled water. Five ml of this solution was added to 10 ml of dichloromethane containing 10~400 μg of macrotetrolide antibiotics. The mixture was shaken vigorously for a minute and then left to stand for a while. The lower dichloromethane layer was removed with a pipette and dehydrated with anhydrous sodium sulfate. Optical density of the dehydrated dichloromethane solution was measured at 377 mμ and the amount of the antibiotic was calculated from the standard calibration curve shown in Fig. 4.

Results and Discussion

It is known that nonactin and monactin form crystalline complexes with Na^+, K^+ and NH_4^+ ions[7]. These antibiotics stimulate oxygen uptake, alkali metal cation

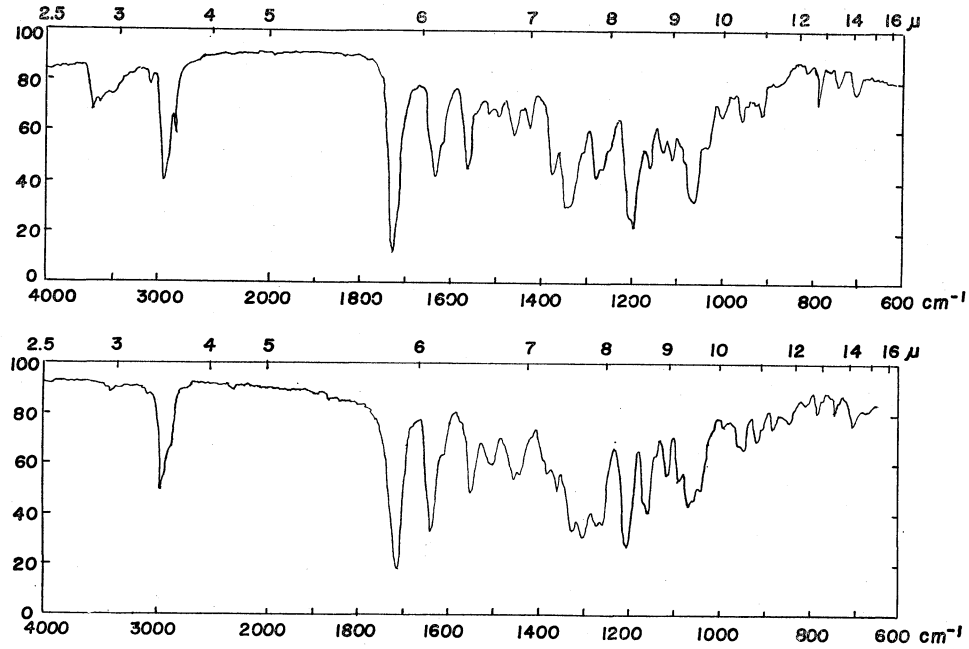
Fig. 1. Structure of macrotetrolide antibiotics



R_1, R_2, R_3, R_4=methyl; nonactin
R_1, R_2, R_3=methyl, R_4=ethyl; monactin
R_1, R_3=methyl, R_2, R_4=ethyl; dinactin
R_1=methyl, R_2, R_3, R_4=ethyl; trinactin
R_1, R_2, R_3, R_4=ethyl; tetranactin

Fig. 2. Infrared absorption spectra of the mixture of tetranactin : sodium picrate (1 : 1) and the complex of tetranactin : sodium picrate (1 : 1) on molar basis.

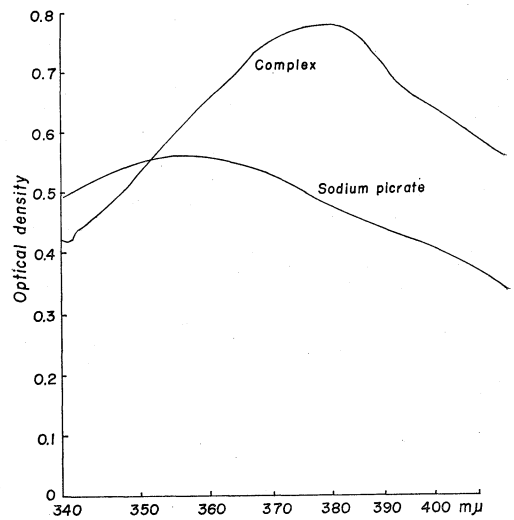
The KBr disc method was used for IR measurement. Tetranactin was added to sodium picrate (1 : 1 on molar basis) and the mixture was ground to a powder. The IR spectrum was taken using this powdered mixture. Then the mixture was dissolved in a small amount of acetone and the resulting solution kept at room temperature for 3 days to allow slow evaporation of the solvent which resulted in needle-shaped crystals of the tetranactin-sodium picrate complex (lower chart). Notice a shift of the carbonyl band of the mixture from 1735 to 1710 cm^{-1} in the complex, and interaction between the Na^+ and the oxygen atoms of the tetrahydrofurane ring bands in the 1250~1050 cm^{-1} region.



transport, and induce ATPase activity in rat liver mitochondria[11]. These facts suggest that once the complexes are formed with the cations, the cations become lipid-soluble and extractable from the aqueous phase into the organic solvent phase accompanied by the concomitant anions[2]. If the anion possesses a chromophore, it should be possible to determine the antibiotics by measuring optical density of the anion extracted from the aqueous phase. Sodium picrate was selected because of its high molar absorptivity[13]. As shown in Fig. 2, the crystalline complex of tetranactin with sodium picrate shows a characteristic shift of the ester carbonyl band (*ca.* 20 cm^{-1}) when the IR spectrum is compared with that of the mixture (1 : 1, on molar basis). The shift is due to the ester carbonyl interaction with Na^+ ion.

Fig. 3. UV spectra of sodium picrate and tetranactin-sodium picrate complex.

Ultraviolet absorption spectra were recorded on a Cary Model 11M U.V. Spectrometer using methanol as solvent. Concentrations of both the mixture and the complex were 0.1 μmol/ml. Tetranactin shows only end-absorption in the range of 200~400 mμ. Notice the characteristic shift due to complex formation.



Complex formation was confirmed by UV spectroscopy. As can be seen in Fig. 3, the UV spectrum of the complex exhibits a bathochromic shift of 20 mμ, indicating that tetranactin forms a complex with sodium picrate in methanol[13].

Table 1 shows the effect of sodium picrate and Na^+ ion on optical density. Addition of sodium hydroxide was expected to shift the equilibrium toward complex formation. However, Na^+ ion has no effect, indicating that the equilibrium is highly

Table 1. Effect of sodium hydroxide and sodium picrate on the complex formation

Sodium picrate (μg/ml)	Sodium hydroxide (N)	Optical density		
		1	2	Mean
50	0.5	0.245	0.246	0.246
〃	1.0	0.238	0.237	0.237
〃	2.0	0.231	0.234	0.232
100	0.5	0.240	0.245	0.243
〃	1.0	0.245	0.244	0.245
〃	2.0	0.250	0.248	0.249
200	1.0	0.245	0.241	0.243
〃	2.0	0.245	0.250	0.248

To 5 ml of dichloromethane containing 9.95 μg/ml of tetranactin were added 5 ml of sodium hydroxide solution and 5 ml of sodium picrate solution. The assay mixture was vigorously shaken for a minute and then kept standing to separate the dichloromethane layer. The lower layer was taken up by pipette and dried over anhydrous sodium sulfate. Optical density of the dichloromethane phase was determined at 377 mμ using a cuvette with 1 cm light path.

Table 2. Effect of various lipids on optical density

Lipids	Lipid concentrations in dichloromethane layer (μg/ml)	Optical density at 377 mμ in the presence of tetranactin (μg/ml)		
		0	10	25
Palmitic acid	1,000	0.085	0.245	0.592
	100	0.026	0.255	0.620
Oleic acid	1,000	0.036	0.273	0.600
	100	0.029	0.260	0.601
Triolein	1,000	0.036	0.273	0.590
Tripalmitin	1,000	0.020	0.242	0.595
Soybean oil	1,000	0.023	0.247	0.595
	10,000	0.019	0.236	0.590
Cholesterol	1,000	0.019	0.235	0.585
	100	0.031	0.236	0.585
Lecithin	100	0.020	0.241	0.593
Control	—	0.000	0.247	0.595

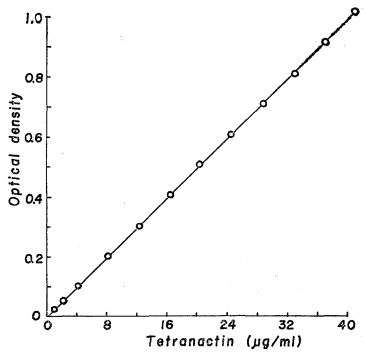
Method; see in the text.

favorable for complex formation. However, sodium hydroxide is added to the assay mixture due to the ability to prevent emulsification. Optical density reached a plateau at a concentration of 200 μg/ml of sodium picrate so that the amount present in the assay mixture is sufficient.

Table 2 shows the effect of some lipids on complex formation. The assay mixture containing fatty acids and phospholipid formed heavy emulsions but by centrifugation separation of clear dichloromethane layer could easily be achieved. These lipids exhibit almost no effect on optical density even at 100 times higher concentration than tetranactin.

Fig. 4. Standard curve for tetranactin

Method ; see in the text



A recovery test was carried out using a fermentation broth containing 24.26 μg of tetranactin. When 41.02 μg of crystalline tetranactin was added to the broth, the assay indicated the presence of 64.55 μg of the antibiotic; the recovery is 98.2 %. Thus, tetranactin can be determined accurately in the assay samples containing various lipids, for example, the fermented broth.

As shown in Fig. 4, the standard calibration curve for the antibiotic is linear from 1 to 40 μg/ml. The equation of the curve is $y=40.3595\,x+0.1050$, where y is μg tetranactin per ml in the dichloromethane layer and x is the optical density.

Dinactin, trinactin and tetranactin were compared by means of this method. As shown in Table 3, the optical densities are proportional to their molar concentrations and, therefore, macrotetrolides can be determined in mixtures simply and accurately.

Table 3. Comparison of each macrotetrolides

Macrotetrolides	Amounts in dichloromethane (μg/ml)	Optical density	Amounts calculated* (μg/ml)	Recovery** (%)	Optical density per μmol
Dinactin	9.344 18.688	0.229 0.443	9.615 18.361	102.9 98.2	18.45
Trinactin	8.544 17.088	0.213 0.415	8.960 17.215	104.8 100.7	19.11
Tetranactin	8.320 16.640	0.203 0.394	8.538 16.344	102.6 98.2	19.02

* Recovery was calculated as follows; the amount calculated from each standard curve per the amount present in dichloromethane layer $\times 100$.

** Molar optical densities were expressed as optical density at 377 mμ of 1 cm light path per 1 μmol of the complex per ml. The molecular formulas of every macrotetrolides are as follows; dinactin $C_{42}H_{68}O_{12}$ mol. wt. 764, trinactin $C_{43}H_{70}O_{12}$ mol. wt. 778, tetranactin $C_{44}H_{72}O_{12}$ mol. wt. 792.

As an alternative to the bioassay with mites and microorganisms, this procedure affords not only a method for the quantitative determination of macrotetrolide antibiotics but also provides a useful tool for isolation and purification of other alkali ion carrier lipid(s) the presence of which is anticipated in all biological membranes.

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