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GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC DETERMINATION OF MACROLIDE ANTIBIOTICS IN BEEF AND PORK USING SINGLE ION MONITORING

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SUMMARY

A gas chromatographic–mass spectrometric (GC–MS) method using single ion monitoring (SIM) is described for the determination of residual macrolide antibiotics, oleandomycin, kitasamycin, spiramycin and tylosin, in beef and pork. For GC–MS determination, oleandomycin is acid hydrolysed to desoleandomycin and acetylated, in the same way as erythromycin. However, for elution from a GC column, the carbon–carbon double bonds in the antibiotics must be hydrogenated to single bonds before acid hydrolysis. Kitasamycin and spiramycin are therefore converted into hydroforocidine acetate and tylosin into hydro-O-mycaminosyl tylonolide acetate, which are determined by GC–MS with SIM.

INTRODUCTION

Antibiotics are applied extensively to food-producing animals to maintain optimum health and promote growth, and their use can leave residues in edible parts. These drug residues have possible direct toxic effects and other safety considerations may include allergic reactions of consumers and induction of resistant bacteria. Therefore, conditions of use in animals must be established for such drugs to ensure human food safety and also efficacy and safety with regard to the animal species[1].

Several macrolide antibiotics, including erythromycin, oleandomycin, kitasamycin, spiramycin and tylosin, are used in agriculture in Japan. They are used both as feed additives for growth promotion and therapeutically, and comprise a large proportion of the total antibiotics used in agriculture. Most antibiotics are assayed by microbiological methods but, although these methods are suitable for screening of drug residues, they are often lengthy, not sufficiently sensitive and lack the specificity and precision required for regulatory purposes. Therefore, a more sensitive, specific and accurate procedure is required for monitoring possible residues in the tissues of food-producing animals.

Thin-layer[2,3] and liquid chromatographic[4,5] methods have been described for the determination of macrolide antibiotics, but their application to residue analyses

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has been limited. More recently, the determination of tylosin residues in foods by liquid chromatography using UV detection has been reported[6], but this method could not be applied to other macrolide antibiotics that do not show pronounced UV absorption.

Gas chromatography–mass spectrometry (GC–MS) with selected ion monitoring (SIM) seems to offer a sensitive and reliable method for the detection and quantitation of residual antibiotics in foods. However, its application is restricted to compounds that can be eluted from a GC column. Macrolide antibiotics are glycosides of 14- or 16-membered lactone derivatives whose molecular weights are relatively large (687–916), and have polar functional groups such as hydroxy, carbonyl and sugars. Hence the elution of these compounds from a GC column after derivatization was considered to be difficult and impractical, if not impossible.

We investigated the removal of sugars from macrolide antibiotics by acid hydrolysis to obtain smaller molecules and to derivatize them in order to elute them from a GC column. Thus, erythromycin was hydrolysed to erythralosamine, which could be determined by GC–MS after acetylation. This method was reported earlier[7]. In this work, this method was applied to other macrolide antibiotics used in agriculture, *viz.*, oleandomycin, kitasamycin, spiramycin and tylosin. Oleandomycin can be determined in the same way as erythromycin after acid hydrolysis to desoleandomycin and acetylation. However, for the determination of kitasamycin, spiramycin and tylosin, it was further necessary to hydrogenate the carbon–carbon double bonds, prior to elution from a GC column, giving hydroforocidine acetate from the first two and hydro-O-mycaminosyl tylonolide (OMT) acetate from the last compound. Detection and quantitation are carried out using single ion monitoring (SIM) at *m/e* 200 (erythralosamine acetate, desoleandomycin acetate) or *m/e* 258 (hydroforocidine acetate, hydro-OMT acetate).

EXPERIMENTAL

Materials and chemicals

Analytical-reagent grade chemicals were used as received. Methanol, ethanol, chloroform, $Na_2HPO_4 \cdot 12H_2O$, $H_2PtCl_6 \cdot 6H_2O$ and $NaBH_4$ were obtained from Wako (Osaka, Japan) and acetic anhydride from Kanto Chemical (Tokyo, Japan). Pyridine (Wako) was dried over potassium hydroxide pellets before use. The solutions were prepared as follows: for 1% Na_2HPO_4 solution, 10.0 g of $Na_2HPO_4 \cdot 12H_2O$ was dissolved in 1000 ml of water; for 10% chloroplatinic acid solution, 1.0 g of $H_2PtCl_6 \cdot 6H_2O$ was dissolved in 10 ml of water; and for sodium chloride solution, 10 g of sodium chloride was dissolved in 100 ml of water. A 1 *M* solution of $NaBH_4$ was prepared fresh just before use by dissolving 3.8 g of $NaBH_4$ in 100 ml of ethanol. If not completely dissolved, the supernatant solution was used. Silica gel (Merck, Darmstadt, F.R.G.) was dried at 140°C for 3 h and stored in a desiccator.

Standard solutions

Stock solution. Amounts of 10 mg of each of the following compounds were dissolved together in 10 ml of methanol in a volumetric flask: erythromycin (Sigma, St. Louis, MO, U.S.A.), oleandomycin (Sigma), spiramycin (Sigma), tylosin (Sigma) and kitasamycin (Toyo Jozo, Shizuoka, Japan).

Working solutions. The stock solution was diluted with methanol to 30, 20, 10, 5, and 1 μg/ml concentrations of each component. The working standard solutions obtained were stored in a refrigerator, protected from light.

Instrumental

A Biotron BT 10 20 350D homogenizer (Biotrona, Küssnacht, Switzerland) and a Model 8-1-W wrist-action shaker (Yayoi, Tokyo, Japan) were used. The glass column for cleanup was 300 mm × 15 mm I.D., with a coarse fritted disc and a PTFE stopcock.

A JMS-D300 gas chromatograph–mass spectrometer (JEOL, Tokyo, Japan) was operated at 70 eV. The system was operated using a JMA-3100 (JEOL) mass data analysis system. The separator temperature was 300°C and the ionization current was 300 μA.

The glass column (0.5 m × 2 mm I.D.) was packed with 5% OV-101 on Chromosorb W H P (80–120) (Hewlett-Packard, Avondale, PA, U.S.A.). After conditioning at 340°C for 5 h the column was treated with Silyl-8 (Pierce, Rockford, IL, U.S.A.). Helium was used as the carrier gas at a flow-rate of 60 ml/min.

Extraction and cleanup

A 10.0 g chopped sample was homogenized and extracted twice with 20 ml of methanol. The extract was centrifuged at 1000 *g* for 10 min and filtered through a fluted paper. To the methanolic extract after washing with 20 ml of *n*-hexane were added 2 ml of 1 *M* sodium hydroxide solution and 30 ml of 1% Na_2HPO_4 solution. The aqueous methanolic solution was extracted twice with 30 ml of chloroform and the combined chloroform was washed with 30 ml of sodium chloride solution and evaporated to dryness using a 100-ml round-bottomed flask and a rotary evaporator at 40–45°C. For the determination of erythromycin and oleandomycin the next step was silica gel column chromatography; for the determination of kitasamycin, spiramycin and tylosin, the next step was hydrogenation.

Hydrogenation

A 0.1 ml volume of 10% chloroplatinic acid solution was added to 10 ml of ethanol in a 100-ml erlenmeyer flask fitted with a PTFE-coated stirring bar. With vigorous stirring, 0.5 ml of 1 *M* ethanolic $NaBH_4$ solution was added all at once and stirred for 1 min, then 0.4 ml of 6 *M* hydrochloric acid was added and stirred for a further 2 min. Platinum black catalyst was produced, and this flask was used as a hydrogenation flask.

A 10 ml volume of ethanol was added to the residue obtained from the extraction and cleanup stages and the residue adhering to the side and bottom of the flask was scraped off with a spatula. This solution was added through a fluted paper to the above hydrogenation flask containing platinum catalyst. The flask and filter-paper were washed with 5 ml of ethanol and the washings were added to the hydrogenation flask. The hydrogenation flask was stirred under a $1 \cdot 10^5$ Pa hydrogen atmosphere for 20 min at room temperature. After hydrogenation, the contents were filtered through a fluted paper, the paper and catalyst were washed with 2 ml of ethanol and the washings were added to the filtrate. A 50 ml volume of water was added to the filtrate, then 2 ml of 5 *M* sodium hydroxide solution were added with ice cooling to make the filtrate alkaline (pH > 9). This aqueous solution was extracted

with 20 ml and then 10 ml of chloroform and the combined chloroform extracts were washed with 20 ml of sodium chloride solution. The chloroform was evaporated using a rotary evaporator at 40–45°C and the residue was used for silica gel column chromatography.

Silica gel column chromatography

The glass column (300 mm × 15 mm I.D.) was packed with 5 g of silica gel with *ca.* 3 g of sodium sulphate on top. The column was pre-washed with 30 ml of chloroform. A 5 ml volume of chloroform was added to the residue obtained from the extraction and cleanup stages or from the hydrogenation step. The residue was dissolved and this chloroform solution was quantitatively transfered to the silica gel column. The flask was rinsed twice with 2 ml of chloroform and the rinsings were added to the column. The column was first eluted with 100 ml of chloroform and the eluate was discarded. Then the column was eluted with 100 ml of chloroform–methanol (2:1) and and the eluate was collected and evaporated using a rotary evaporator at 40–45°C. This was followed by acid hydrolysis and acetylation.

Acid hydrolysis and acetylation

For the determination of erythromycin, oleandomycin, kitasamycin and spiramycin, 15 ml of 0.3 *M* hydrochloric acid were added to the residue obtained by silica gel column chromatography and the residue was scraped with a spatula to remove and dissolve the residue. The mixture was warmed on a water-bath at 50°C for 1 h, then cooled in an ice-bath. A 1.2–1.3 ml volume of 5 *M* sodium hydroxide solution was added to the hydrolysis mixture to make it alkaline (pH > 9) and the aqueous solution obtained was extracted with 20 ml and then 10 ml of chloroform. The combined chloroform extracts were washed with 20 ml of sodium chloride solution and dried over sodium sulphate. The chloroform was evaporated using a 50-ml pear-shaped or round-bottomed flask using a rotary evaporator at 40–45°C and the last trace of solvent was evacuated with a rotary oil pump for 10 s. A 500 μl volume of dry pyridine and 250 μl of acetic anhydride were added to dissolve the residue and the flask was stoppered tightly. The mixture was allowed to stand at room temperature overnight with occasional swirling, and was then ready for injection into the GC–MS system. The analysis must be completed within 3 days.

For the determination of tylosin, acid hydrolysis conditions (refluxing for 30 min in 0.3 *M* hydrobromic acid was used instead of at 50°C with 0.3 *M* hydrochloric acid. The remaining procedure was the same as described above.

Preparation of standard solutions for calibration graphs

Erythromycin and oleandomycin. Volumes of 1 ml of working standard solutions containing 10, 5 or 1 μg/ml of antibiotics were pipetted into 50-ml round-bottomed flask and the solvent was evaporated with a rotary evaporator. A 15-ml volume of 0.3 *M* hydrochloric acid was added and the solution was treated as described under *Acid hydrolysis and acetylation.*

Kitasamycin, spiramycin and tylosin. Volumes of 1 ml of working standard solutions containing 30, 20 or 10 μg/ml of antibiotics were hydrogenated as described under *Hydrogenation* and then treated as described under *Acid hydrolysis and acetylation.* For kitasamycin and spiramycin, hydrolysis was carried out at 50°C in 0.3

M hydrochloric acid for 1 h was used, and for tylosin, refluxing with 0.3 *M* hydrobromic acid for 30 min was used.

Gas chromatographic–mass spectrometric analysis

The GC column was connected to the ion source of the mass spectrometer and the instrument parameters were adjusted. The magnetic and electronic fields were calibrated using perfluorokerosene as a standard.

For the determination of erythromycin and oleandomycin, the GC column temperature was adjusted to 275°C and the detection ion in SIM was set at *m/e* 200. For the determination of kitasamycin, spiramycin and tylosin, the GC column temperature was adjusted to 290°C and the detection ion in SIM was set at *m/e* 258. The mass valve was opened 50 s after the start of a chromatographic run.

A 2–5 μl volume of each standard solution was injected into the GC–MS system and a calibration graph was constructed by plotting the peak area against the amount of the antibiotic. A 2–5 μl volume of sample solution was subsequently injected into the GC–MS system and the compounds were identified from the retention times of the peaks and from the peak shapes. The amount of each antibiotic contained in the sample solution was calculated by comparison of the peak area with the calibration graph. The concentration of each antibiotic in the sample was calculated by dividing the amount of antibiotic by the amount of the sample (10.0 g).

RESULTS AND DISCUSSION

Extraction and cleanup

For the extraction of antibiotics from sample tissues, methanol is used because of its high solubility towards macrolide antibiotics and the ease of permeation into sample tissues. The co-extractives from a 10 g sample of beef and pork amount to *ca.* 200–300 mg. In order to remove co-extractives and to clean up the antibiotics, washing of the methanol extract with *n*-hexane and partition between chloroform and phosphate buffer are used. These treatments are the same as those used previously in the determination of erythromycin[7]. After these treatments, the amount of extracted material is reduced to *ca.* 70–150 mg, which makes the next procedure (silica gel column chromatography or hydrogenation of carbon–carbon double bonds) easy to perform. Cleanup by silica gel column chromatography is also necessary, otherwise interfering peaks are observed on the SIM chromatograms at *m/e* 200 and *m/e* 258.

Gas chromatographic–mass spectrometric determination

At first, for elution through a GC column after derivatization, we intended to use the aglycones of the macrolide antiotics. However, it has been reported[8–10] that the glycoside linkages of amino sugars, desosamine (erythromycin, oleandomycin) and mycaminose (kitasamycin, spiramycin, tylosin) resist acid hydrolysis much more than those of neutral sugars; the conditions normally required for the removal of an amino sugar from a macrolide antibiotic induce extensive destruction of the aglycone. These facts were confirmed by our experiments, so we decided to use compounds that can be obtained by mild acid hydrolysis of macrolide antibiotics. These compounds are erythralosamine, desoleandomycin, forocidine and O-mycaminosyl ty-

lonolide (OMT). In these compounds, an amino sugar is bonded to the lactone aglycone. Kitasamycin and spiramycin gave the same compound, forocidine, after acid hydrolysis, so differentiation of these two antibiotics is not possible with this proposed method.

In order to simplify the analytical procedures, the same conditions for acid hydrolysis of heating at 50°C in 0.3 *M* hydrochloric acid for 1 h were used for all the macrolide antibiotics except tylosin. With tylosin, hydrolysis to desmycosin (removal of mycarose only) was accomplished under mild conditions (0.1 *M* hydrochloric acid, room temperature, 2 h), but the complete transformation of desmycosin to OMT was difficult. A mixture of desmycosin and OMT was obtained after refluxing tylosin in 0.1 *M* sulphuric acid for 8 h, although it has been reported[9,11] that prolonged refluxing of tylosin in dilute hydrochloric or sulpuric acid gives OMT. We found that refluxing with 0.3 *M* hydrobromic acid for 30 min was sufficient for complete hydrolysis to OMT. Hence the hydrolysis conditions for tylosin differ from those for the other macrolide antibiotics.

The derivatization reactions generally used in GC determinations are silylation, acetylation and ether formation. We chose to utilize acetyl derivatives, because they are easily prepared and reasonably stable and the increase in molecular weight on derivatization is not so large. The sensitivity of GC–MS decreases at high *m/e*, so high molecular weights are undesirable. Acetylation was easily accomplished by standing the hydroxyl compound in a mixture of acetic anhydride and pyridine at room temperature.

The conditions for the GC of the acetylated derivatives of the acid-hydrolysed products were investigated using flame ionization detection (FID) and it was found that desoleandomycin acetate gave relatively sharp, clear peaks on the chromatograms, as did erythralosamine acetate[7]. However, the acetates of forocidine and OMT did not give clear peaks, but broad, diffuse curves on the chromatograms. Also, forocidine acetate and OMT acetate did not show clear peaks on the GC–MS total ion chromatograms, but only raised baselines at a half of full-scale. These findings indicate that forocidine acetate and OMT acetate decomposed on the GC column at high temperatures. Two additional moieties, a formyl group and carbon–carbon double bonds, are present in forocidine and OMT. Therefore, we hydrogenated the double bonds of forocidine and OMT in ethanol in the presence of a palladium–carbon catalyst under a $1 \cdot 10^5$ Pa hydrogen atmosphere. The hydrogenated products were acetylated and injected into the GC–FID system. In these instances, sharp, clear peaks were obtained on the chromatograms. Hence we concluded that in order to elute acetates of forocidine or OMT, it is necessary to hydrogenate their carbon–carbon double bonds. The yields of hydrogenation were low when small amounts were reduced, and none of products were obtained when microgram amounts were hydrogenated over a palladium catalyst. Therefore, a platinum catalyst was tried instead of palladium and it gave sufficient yields of reduction products at the microgram level and below. Various synthetic methods for platinum catalysts have been reported[12], the procedure of Brown and Brown[13] was employed because it produces a very active platinum catalyst by an easy, simple procedure.

In Fig. 1 the peak heights of the acetylation products are plotted against reaction time at room temperature. For erythralosamine[7] and desoleandomycin, the peak of a major product obtained after 30 min decreased gradually and another peak

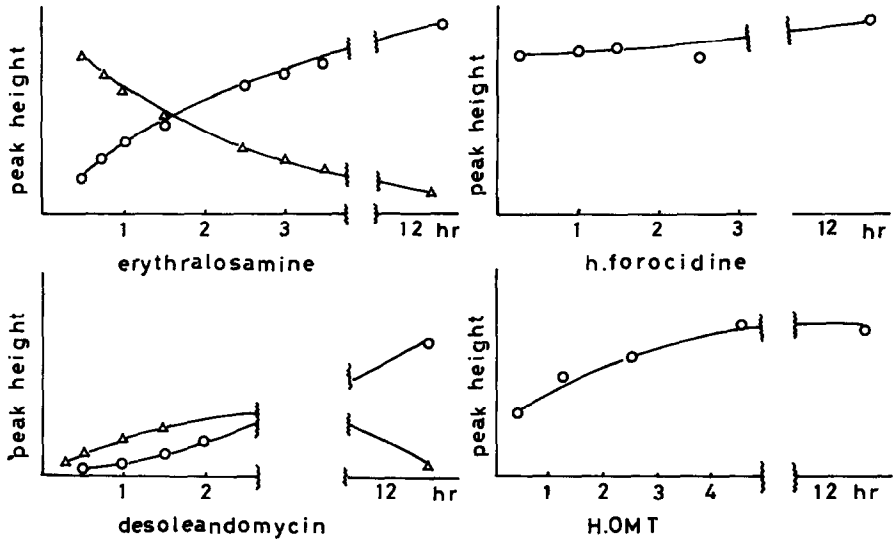

Fig. 1. Reaction time *vs.* peak heights of acetylated products of erythralosamine, desoleandomycin, hydroforocidine and hydro-OMT in acetic anhydride–pyridine (1:2) at room temperature.

increased. The hight of the latter peak reached a maximum after reaction for over 10 h, so an overnight reaction is used for acetylation. The amount of acetylated products decreased to about half the original amount after keeping for 2 weeks in a refrigerator, so GC–MS determination must be carried out within 2–3 days after acetylation.

Figs. 2 and 3 show the electron impact mass spectra of acetylated erythralosamine, desoleandomycin, hydroforocidine and hydro-OMT. Pronounced peaks at *m/e* 200 (Fig. 2) and *m/e* 258 (Fig. 3) are observed. The peak at *m/e* 200 is common to macrolide antibiotics with a 14-membered lactone ring and the peak at *m/e* 258 is common to those with a 16-membered lactone ring. These ions are considered to be the amino sugars, desosamine acetate and mycaminose acetate (Fig. 4), which could not be eliminated from the aglycones by mild acid hydrolysis. In order to obtain high sensitivity, these ions are used for detection in GC–MS with SIM and by the use of these two ions all the macrolide antibiotics considered here can be determined.

Kitasamycin and spiramycin gave the same compound, forocidine, on acid hydrolysis. Hence in this method, the detection of hydroforocidine acetate implies that residues of kitasamycin or spiramycin are present in the sample, but it is not possible to differentiate between them.

Fig. 5 shows the SIM chromatograms (*m/e* 200) of blank samples and extracts from spiked samples, and Fig. 6 shows the SIM chromatograms (*m/e* 258) of blank samples and extracts from spiked samples. No interfering peaks are observed on the SIM chromatograms of blank samples, and each compound derived from the macrolide antibiotics can be clearly identified. The peak shape is not as sharp as that of erythralosamine acetate[7], probably because a standard compound of each macrolide antibiotic consists of several components, and isomerizations may occur during acid

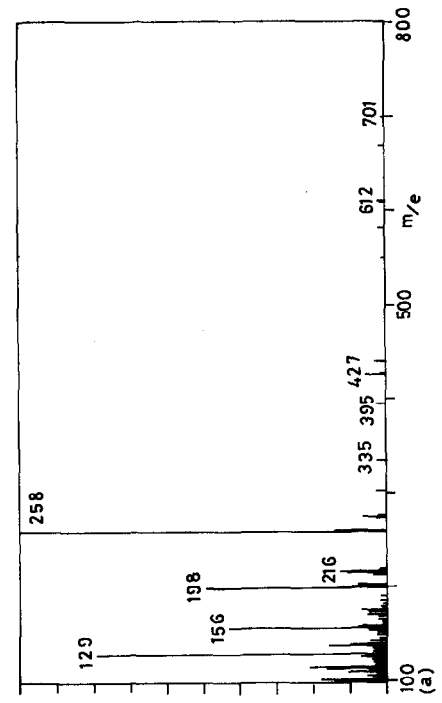

Fig. 2. Electron impact mass spectra of (a) erythralosamine acetate and (b) desoleandomycin acetate (70 eV).

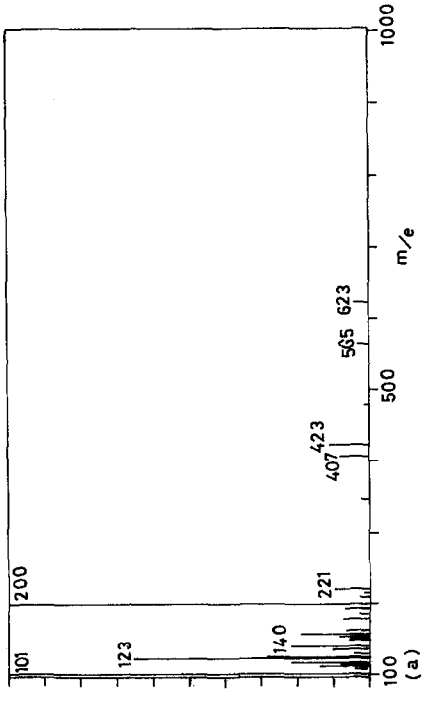

Fig. 3. Electron impact mass spectra of (a) hydroforocidine acetate and (b) hydro-OMT acetate (70 eV).

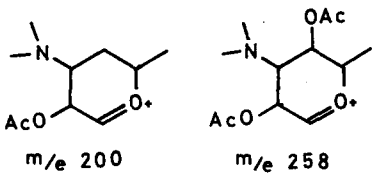

Fig. 4. Structures of fragment ions of *m/e* 200 and 258 in electron impact mass spectra.

hydrolysis, thus making the composition more complex. However, each macrolide antibiotic can be identified from the retention times and peak shapes. The peak(s) of desoleandomycin acetate eluted from 2.3 to 4.5 min on a 5% OV-101 column (0.5 m) at 275°C (Fig. 5). Hydroforocidine acetate eluted from 1.5 to 3.5 min and hydro-OMT acetate from 4.0 to 7.5 min on a 5% OV-101 column (0.5 m) at 290°C (Fig. 6). The SIM mode provided integrated peak area information for specific retention time periods. The peak areas for the above retention times were used for quantitation. Plots of SIM responses (peak areas) against the amount of standards injected were linear over the whole range (desoleandomycin acetate 5–100 ng, hydroforocidine acetate. 20–140 ng, hydro-OMT acetate 50–800 ng) and the correlation coefficients were 0.98-0.99.

Recovery experiments and detection limit

Beef and pork tissues have been previously analysed for macrolide antibiotics using the proposed method, and tissues in which no macrolide antibiotics were detected were used for revcovery experiments. Table I shows the recoveries and coefficients of variation (C.V.s). The recoveries (70–90%) and C.V.s for oleandomycin are satisfactory, but those for kitasamycin, spiramycin and tylosin are not sufficiently good, which probably resulted form the hydrogenation process before determination.

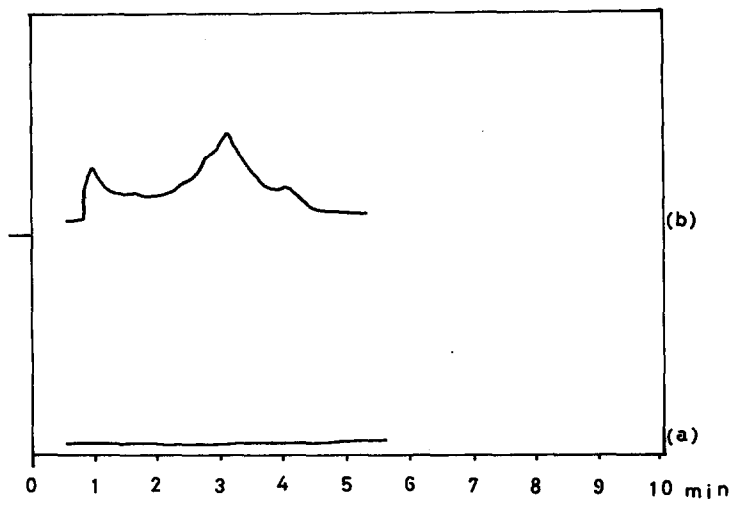

Fig. 5. SIM chromatograms at *m/e* 200 of (a) extract from blank sample (pork) and (b) extract from pork spiked with 1 ppm of oleandomycin. GLC column conditions: 5% OV-101 (0.5 m), 275°C.

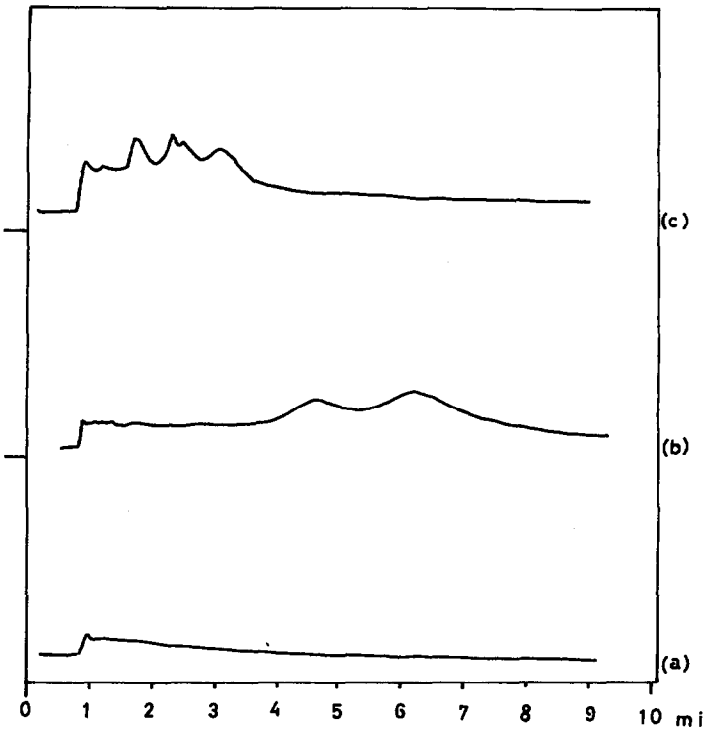

Fig. 6. SIM chromatograms at *m/e* 258 of (a) extract from blank sample (beef), (b) extract from beef spiked with 3 ppm of tylosin and (c) extract from beef spiked with 1 ppm of spiramycin. GLC column conditions: 5% OV-101 (0.5 m), 290°C.

The C.V.s are high for kitasamycin, spiramycin and tylosin, so the results are semi-quantitative, but the identification of the residual antibiotics by this means is more reliable than that by microbiological methods.

The detection limits were determined by analyzing spiked samples at various concentrations using the proposed method. The results (concentrations in samples) were 0.1 ppm for oleandomycin and 0.5 ppm for kitasamycin, spiramycin and tylosin.

TABLE I

RECOVERIES OF MACROLIDE ANTIBIOTICS FROM BEEF AND PORK SPIKED AT 1 ppm CONCENTRATION

Antibiotic	*Beef*		*Pork*	
	*Recovery (%)**	*C.V. (%)*	*Recovery (%)**	*C.V. (%)*
Oleandomycin	76.2	2.1	69.7	12.8
Kitasamycin	59.2	28.4	52.8	25.4
Spiramycin	61.6	4.5	69.6	20.9
Tylosin	48.2	20.0	46.9	21.7

*The recovery data are based on three samples.

The low detection limit of erythromycin[7] is due to the effect of the sharp peak shape of erythralosamine acetate. The detection limits of the other antibiotics and the accuracy of determination may be improved if a GC column with a higer resolving power, such as a capillary column, is used, and application of such a column is now under investigation. Although kitasamycin and spiramycin cannot be differentiated by this method, it is a practical, specific, physico-chemical method that can be used as a routine screening method.

REFERENCES

1 R. C. Livingston, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 966.
2 J. Majer, in J. J. Weinstein and G. H. Wagman (Editors), *Antibiotics, Isolation and Purification (Journal of Chromatography Library*, Vol. 15), Elsevier, Amsterdam, 1978, p. 273.
3 J. Majer, J. R. Martin, R. S. Egan and J. W. Corcoran, *J. Am. Chem. Soc.*, 99 (1977) 1620.
4 J. H. Kennedy, *J. Chromatogr. Sci.*, 16 (1978) 492.
5 S. Bhuwaphathanapun and P. Gray, *J. Antibiot.*, 30 (1977) 673.
6 Y. Hoshino, M. Horie, N. Nose and H. Iwasaki, *J. Food Hyg. Soc. Jpn.*, 25 (1984) 430.
7 K. Takatsuki, S. Suzuki, N. Sato, I. Ushizawa and T. Shoji, *J. Assoc. Off. Anal. Chem.*, in press.
8 E. H. Flynn, M. V. Sigal, Jr., P. F. Wiley and K. Gerzon, *J. Am. Chem. Soc.*, 76 (1954) 3121.
9 R. B. Morin, M. Gorman, R. L. Hamill and P. V. Demarco, *Tetrahedron Lett.* (1970) 4737.
10 S. Masamune, Y. Hayase, W. K. Chan and R. L. Sobeczak, *J. Am. Chem. Soc.*, 98 (1976) 7874.
11 R. B. Morin and M. Gorman, *Tetrahedron Lett.*, (1964) 2339.
12 L. F. Fieser and M. Fieser, *Reagents for Organic Syntheses*, Wiley, New York, 1967, p. 890.
13 H. C. Brown and C. A. Brown, *J. Am. Chem. Soc.*, 84 (1962) 1492.