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Simultaneous determination of five macrolide antibiotics in meat by high-performance liquid chromatography

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Abstract

A simple and rapid method using high-performance liquid chromatography (HPLC) for the simultaneous determination of five macrolides (josamycin, kitasamycin, mirosamicin, spiramycin and tylosin) in meat has been developed. The drugs were extracted with 0.3% metaphosphoric acid–methanol (7:3, v/v), and the extracts were cleaned up on a Bond Elut SCX (500 mg) cartridge. The HPLC separation was performed on a Puresil $5C_{18}$ column (150×4.6 mm I.D.) with a gradient system of 0.025 *M* phosphate buffer (pH 2.5)–acetonitrile as the mobile phase at a flow-rate of 1.0 ml/min. The drugs were detected at 232 nm for josamycin, kitasamycin, mirosamicin and spiramycin, and 287 nm for tylosin. The calibration graphs were rectilinear from 2.5 to 100 ng for each drug. The recoveries at the level of 1.0 μg/g were 70.8–90.4%, and detection limits were 0.05 μg/g for each drug.

Keywords: Food analysis; Macrolides; Antibiotics

1. Introduction

Macrolide antibiotics are a very important class of antibacterial compounds widely used in medical and veterinary practices. Josamycin (JM), kitasamycin (KT), mirosamicin (MRM), spiramycin (SPM) and tylosin (TS) belong to the class of 16-membered macrolide antibiotics. These are highly active against a wide range of Gram-positive bacteria, *Mycoplasma* and *Chlamydia*. The macrolides are the most effective medicine against diseases produced by *Mycoplasma* species [1]. These drugs are absorbed well after oral administration and are distributed extensively in tissues, especially lungs, liver and kidneys, with high tissue/plasma ratios [2]. Therefore, these macrolides have been widely used in the rearing of food-producing animals to prevent and treat diseases. Of these compounds, MRM and TS are used exclusively in veterinary drugs.

Incorrect use of these drugs may leave residues in edible tissues. Antibiotic residues may have direct toxic effects on consumers, e.g., allergic reactions in hypersensitive individuals, or may indirectly cause problems through the induction of resistant strains of bacteria [3]. Therefore, simple and reliable analytical methods are required to monitor these drug residues in edible tissues of livestock animals. Generally, the determination of antibiotics, including JM, KT, MRM, SPM and TS, is mainly carried out by microbiological assays [4–6]. These assays excel as a qualitative means by which samples may be screened for residual amounts of antibacterial sub-

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stances. However, the assays tended to lack specificity, and their use involves difficulty in confirming what kinds of drugs remain in the animal tissues. In order to overcome these problems, chemical analyses such as high-performance liquid chromatographic (HPLC) techniques have been used for the determination of macrolide antibiotics [7–16]. However, there are no methods for the simultaneous determination of the five macrolides by HPLC.

This paper describes a simple, rapid and reliable HPLC method for the simultaneous determination of JM, KT, MRM, SPM and TS at 0.05 μg/g in chicken, swine and cattle tissues.

2. Experimental

2.1. Materials and reagents

JM, KT, MRM, SPM and TS were kindly supplied by Yamanouchi Pharmaceuticals (Tokyo, Japan), Asahi Chemical Industry (Tokyo, Japan), Asahi Chemical Industry, Kyowa Hakko Kogyo (Tokyo, Japan) and Takeda Pharmaceuticals (Osaka, Japan), respectively.

Bond Elut SCX (500 mg) cartridges were purchased from Varian (Harbor City, CA, USA). The cartridges were conditioned by washing with 5 ml of methanol and then 10 ml of 0.1 *M* potassium dihydrogenphosphate (pH 4.4) before use. Hyflo Super-Cel was obtained from Johns-Manville (Denver, CO, USA). Antibiotic medium 5 (Difco; Detroit, MI, USA) was used for the bioassay of antibacterial activity. Other chemicals were of analytical-reagent or HPLC grade. Deionized or distilled water was used throughout all experiments.

Mobile phase A was 0.025 *M* phosphate buffer solution (2.5 g of sodium dihydrogenphosphate dihydrate and 0.65 ml of 85% phosphoric acid dissolved in 1 l of distilled water, pH 2.5), while mobile phase B was 0.025 *M* phosphate buffer (pH 2.5)–acetonitrile (60:40, v/v). The phosphate buffer solution was filtered through a Millipore GS 0.22-μm filter (Milford, MA, USA).

2.2. Preparation of standard solutions

Stock standard solutions of JM, KT, MRM, SPM and TS were prepared by dissolving 50 mg of each compound in 50 ml of methanol. The standard solutions were kept at 5°C in amber glass vessels and were stable for up to 3 months. Working standard solutions were prepared by diluting the stock solution with 0.05 *M* sodium dihydrogenphosphate (pH 4.5)–acetonitrile (7:3, v/v). The working standard solutions were stored in the refrigerator and were stable for up to 3 days.

2.3. Apparatus

Experiments were carried out using a Waters (Milford, MA, USA) Model LC Module I liquid chromatograph system (Model 600 pump, Model 717 autosampler, Model 486 spectrophotometric detector). All data collection and calculations were performed using a Waters Model 825 data station. The separation was performed on a Puresil $5C_{18}$ column (5 μm, 150×4.6 mm I.D, Waters) with a gradient system of 0.025 *M* phosphate buffer (pH 2.5)–acetonitrile as the mobile phase at a flow-rate of 1.0 ml/min. The gradient was initiated with 40% eluent B followed by a linear increase to 100% eluent B over 16 min. The column temperature was kept at 35°C. The extracts were analyzed by a UV detector with wavelength programming. During the 9 min of the analysis, the wavelength was set at 232 nm. After 9 and 11 min of the analysis, the wavelength was changed to 287 and 232 nm, respectively.

The other instruments used were a Model 330 spectrophotometer (Hitachi, Tokyo, Japan) and a Model NS-50 Physcotron homogenizer (Niti-on, Chiba, Japan).

2.4. Sample preparation

The sample preparation was done as follows based on a previous paper [15]. A 5-g sample was homogenized at high speed for 2 min with 100 ml of 0.3% metaphosphoric acid–methanol (7:3, v/v) used as a deproteinizing extractant. The homogenate was filtered through ca. 2 mm of Hyflo Super-Cel coated on a suction funnel. In the case of a liver or kidney sample, several grams of Hyflo Super-Cel were added to the homogenized solution before the filtration. After slightly mixing, the obtained mixture was filtered. The filtrate was evaporated under reduced

pressure at 45°C. Evaporation was interrupted when ca. 20 ml of solution remained in the flask. The flask contents were applied to a Bond Elut SCX cartridge. After washing with 10 ml of distilled water and 5 ml of 0.1 *M* dipotassium hydrogenphosphate (pH 8.9), respectively, the cartridge was eluted with 10 ml of methanol. The eluate was evaporated to dryness under reduced pressure at 45°C, and the residue was dissolved in 1 ml of 0.05 *M* sodium dihydrogenphosphate (pH 4.5)–acetonitrile (7:3, v/v). A 10-μl sample of the solution was then injected into the HPLC system.

2.5. Calibration graphs

Standards at concentrations of 0.25, 0.5, 1.0, 2.0, 5.0 and 10 μg/ml of JM, KT, MRM, SPM and TS were prepared from stock standard solution. A 10-μl volume of these solutions was injected into the column. Calibration graphs were obtained by measurement of peak areas.

2.6. Microbiological assay

The antibacterial activities of JM, KT, MRM, SPM and TS were measured using a paper disk method with *Bacillus subtilis* ATCC 6633, *Bacillus cereus* var. *mycoides* ATCC 11778 and *Micrococcus luteus* ATCC 9341 as the test organisms. The plates were incubated at 30°C for 18 h. Calibration graphs were constructed by plotting the diameters of the inhibition zones against the logarithm of the drug concentrations. The assay procedure was carried out according to the official method provided by the Ministry of Health and Welfare, Japan [6].

3. Results and discussion

3.1. Indicator component

Sixteen-membered macrolides are mostly produced as complex mixtures of related components [17]. SPM consists of three components, spiramycin I, II and III. TS is composed of four components, tylosin A, B, C and D; tylosin A is the main and most important component. Similarly, KT consists of several components, leucomycin (LM) A1, A3–9 and A13, with leucomycin A5 as the major component. It is difficult to monitor all components of these macrolides in animal tissues. When the residual level is high enough to detect each component, it is preferable to monitor each component. However, the residual drug level in animal tissues is actually too low. Therefore, it is thought to be a practical measure to assume the main component of these drugs to be an indicator to evaluate the residual level.

The Japan Antibiotics Drugs Standards were revised overall in 1990 [18]. In fact, the old reference standards comprised many components; however, a compound was specified as a single component in order to provide the reference standard with a constant quality of good reproducibility.

Standard SPM comprised spiramycin I (SPM-I), standard TS is tylosin A(TS-A), and standard KT comprised leucomycin A5 (LM-A5) as a single component. However, SPM, TS and KT preparations consist of many components. Previously, we reported the determination methods for SPM, TS and KT in preparations by HPLC [8,10,16]. The compositions of SPM, TS and KT preparations were then examined using the previous methods. As shown in Table 1, SPM preparations included about 70% SPM-I. TS preparations included about 90% TS-A (Table 2), and KT preparations included about 50% or more LM-A5 (refer to the previous paper [16]). The results were almost the same as those of the previous report [19–21]. Accordingly, SPM-I, TS-A and LM-A5 were indicators for SPM, TS and KT residues, respectively.

Table 1
Determination of spiramycin components in pharmaceutical preparations

Sample	Composition (%)			
	SPM-I	SPM-II	SPM-III	Others
1	69.8	8.5	11.4	10.3
2	72.1	8.5	12.5	6.9
3	71.5	7.9	9.7	10.9
4	69.2	9.1	11.6	10.1
5	71.0	7.2	12.0	9.8
Reference 1	95.7	0.5	0.6	3.2
Reference 2[a]	70.0	8.4	12.1	9.5
Reference 3[a]	69.3	7.8	12.1	10.8

[a]These were old reference preparations of spiramycin.

Table 2
Determination of tylosin components in tylosin preparations

Sample	Composition (%)				
	Tylosin A	Tylosin B	Tylosin C	Tylosin D	Others
1	89.7	3.4	1.6	3.2	2.1
2	90.1	2.8	0.6	2.9	3.6
3	93.0	2.0	1.5	1.2	2.3
4	90.7	3.0	0.6	2.9	2.8
5	91.1	3.3	0.9	2.7	2.0
Reference 1	93.3	3.3	0.1	2.3	1.0
Reference 2[a]	88.1	3.2	0.7	3.9	4.1
Reference 3[a]	75.7	5.3	8.7	6.1	4.2

[a]These were old reference preparations of tylosin.

Next, the compositions of JM (leucomycin A3) and MRM (mycinamicin II) preparations, defined as the single component, were determined. As a result, each preparation included 90% or more JM and MRM.

3.2. Chromatographic conditions

JM, KT, MRM, SPM and TS have relatively strong UV absorption [15,17], so that UV detection is sufficiently sensitive in the determination of these macrolides. In the HPLC–UV analysis of chemicals, it is extremely important that an appropriate detection wavelength be selected. Without any interference from contaminants, the maximum absorption wavelength of the compound to be analyzed is generally employed as the wavelength of detection. The five macrolides were dissolved in 0.05 *M* phosphate buffer (pH 4.5)–acetonitrile (7:3, v/v) and their UV spectra were measured. The UV absorption maxima were in the vicinity of 230 nm for JM, KT, MRM and SPM, and 287 nm for TS. To detect these drugs was difficult at the same fixed wavelength. Therefore, the drugs were detected by a UV detector with wavelength programming.

A disadvantage of HPLC of basic substances on silica-based reversed phases is peak tailing due to interaction with residual silanols on the silica gel. Similar to a number of other macrolide antibiotics, JM, KT, MRM, SPM and TS, which are basic molecules containing amino sugar(s) in their structure, are strongly affected by silanol groups remaining in the column packing material [22]. Therefore, the HPLC column used was a Puresil $5C_{18}$, an end-capped ODS column based on pure silica gel.

The choice of adequate conditions for the HPLC procedure is governed by the ionizable groups, amino sugar(s), of the macrolide antibiotics. Silica-based C_{18} columns are overwhelmingly used with mixtures of buffer and acetonitrile (or methanol) as a mobile phase. Optimum conditions were sought by varying the mobile phase pH and the concentration ratio of sodium phosphate buffer to acetonitrile in the mobile phase. The capacity factor (k') decreased markedly for the five macrolides as the acetonitrile concentration rose. However, there were large differences in the affinity for the column among the macrolides, so that analysis by the isocratic elution system was difficult. The gradient elution method was then adopted in this experiment.

Next, the effect of the pH of the mobile phase on peak shape and retention times of the drugs was studied. The simple aqueous acetonitrile mobile phase has a pH of around 7, which, it is thought, would dissociate any residual silanols to weakly anionic species that could strongly retain the basic macrolides. To prevent this, the mobile phase pH was adjusted to be acidic. The asymmetry and retention time of the peaks increased with increasing pH in the range of 2.0–5.0. As a result of the above investigations, eluent A, 0.025 *M* phosphate buffer (pH 2.5), and eluent B, 0.025 *M* phosphate buffer (pH 2.5)–acetonitrile (60:40), were chosen as the mobile phase. Fig. 1 shows typical chromatograms of the standard mixture of the macrolides obtained under these conditions.

Generally, macrolide antibiotics are not stable in

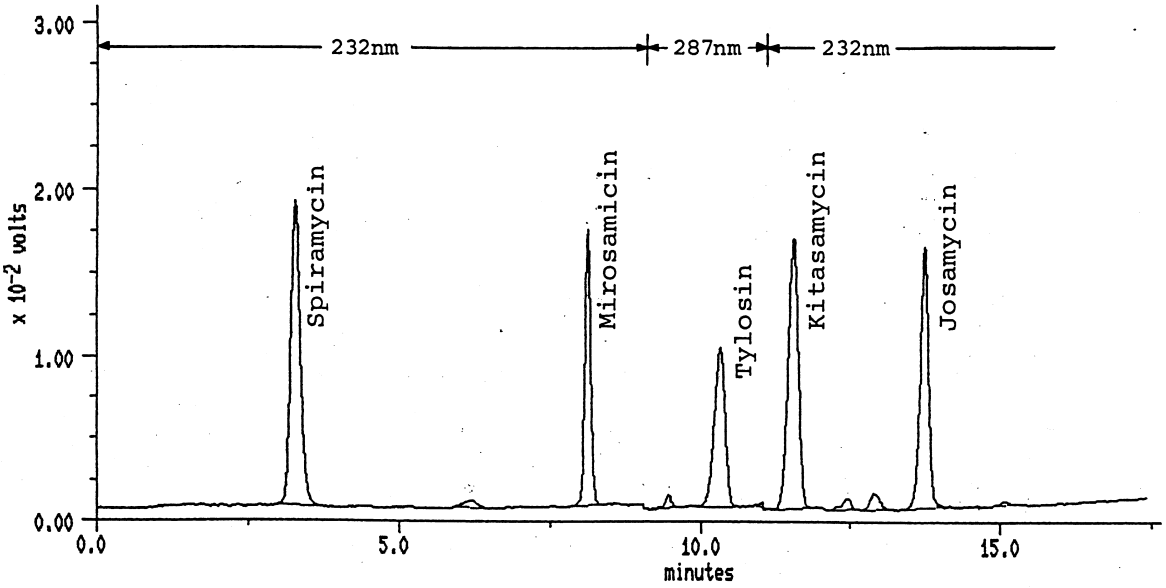

Fig. 1. Typical chromatogram of standard mixture (each 50 ng). LC conditions: column, Puresil $5C_{18}$; mobile phase A, 0.025 *M* phosphate buffer (pH 2.5); mobile phase B, 0.025 *M* phosphate buffer (pH 2.5)—acetonitrile (60:40, v/v); gradient profile, 40% B (0 min) to 100% B (16 min); flow-rate, 1.0 ml/min; detection, wavelength programming.

acidic solutions. However, investigations of the stabilities of 5.0 μg/ml of the five macrolides in the mobile phase at 35°C for 30 min demonstrated that they were stable under these conditions. Consequently, the use of an acidic HPLC mobile phase did not present a problem.

The gradient elution adopted in the present study has a relatively lower repeatability than isocratic elution. Thus, the repeatability of the retention time and of the peak area of the five macrolides was examined under these conditions (Table 3). Satisfactory results were obtained under these conditions: relative standard deviations (R.S.D.) for retention time of 0.3% or less, and R.S.D. of peak area of 0.5–2.5%.

Table 3
Repeatability of retention time and peak area of macrolide antibiotics

Antibiotics	Retention time (min)		Peak area, R.S.D. (%)
	Mean±S.D.	R.S.D. (%)	
Spiramycin	3.23±0.01	0.31	2.49
Mirosamicin	8.02±0.01	0.12	1.70
Tylosin	10.20±0.02	0.20	1.61
Kitasamycin	11.52±0.02	0.17	2.32
Josamycin	13.73±0.01	0.07	0.48

$n=6$

3.3. Clean-up

The extract from a tissue sample contains many diverse compounds in addition to the possible traces of the target analytes. To exclude these interfering substances, a variety of techniques may be employed.

A clean-up method for lipophilic compounds, such as macrolide antibiotics, that has been used for many years is the liquid–liquid partitioning method [7–10]. These drugs are generally extracted from the biological matrix into organic solvents (typically employing chloroform, or dichloromethane) at a pH where the ionization of their basic function, amino sugar, is suppressed. For the clean-up of SPM and TS contained in meat, we employed the liquid–liquid partition method [8,10]. Therefore, we discussed a clean-up procedure by liquid–liquid partitioning using dichloromethane. However, this method was found to be applicable for the clean-up of muscle samples, but was not suitable for liver and kidney samples due to extensive emulsion formation that

prevented the elimination of interfering substances. In addition, chlorinated solvents may pose both safety concerns and create expensive waste-disposal problems.

Recently, a solid-phase extraction (SPE) method has been extensively applied in the clean-up of biological fluids, such as serum and urine, prior to being quantitatively analyzed for trace amounts of macrolide antibiotics [23–25]. The most widely used solid-stationary phase for extraction and clean-up is octadecyl silane bonded to silica gel, called C_{18} or ODS. Thus we examined whether or not the C_{18} cartridge was applicable to the cleaning up of the five macrolides in animal tissues. Accordingly, the macrolides were strongly held by the C_{18} cartridge. In the case of liver and kidney samples containing many contaminants compared with the biological fluids, however, the contaminants could not be completely eliminated by cleaning up with the use of the C_{18} cartridge.

In a previous paper [15], we reported the determination of MRM in animal tissues where samples were extracted with metaphosphoric acid–methanol, and the clean-up procedure used a cartridge packed with a silica-based cation-exchange phase (Bond Elut SCX). All five macrolides are basic and lipophilic compounds. Therefore, we evaluated the use of such a method for the simultaneous determination of JM, KT, MRM, SPM and TS. Using this method, a good recovery was obtained without any interference from coexisting substances, as shown in Fig. 2.

3.4. Recovery

Linear calibration graphs were obtained from 2.5 to 100 ng (equivalent to 0.05–2.0 μg/g) for the five macrolides. Table 4 summarizes the recoveries of the drugs from samples of chicken muscle and liver, swine muscle, liver and kidney, and cattle muscle and liver fortified with 1.0 μg/g. Greater than 70% overall mean recoveries and within 10% R.S.D. were

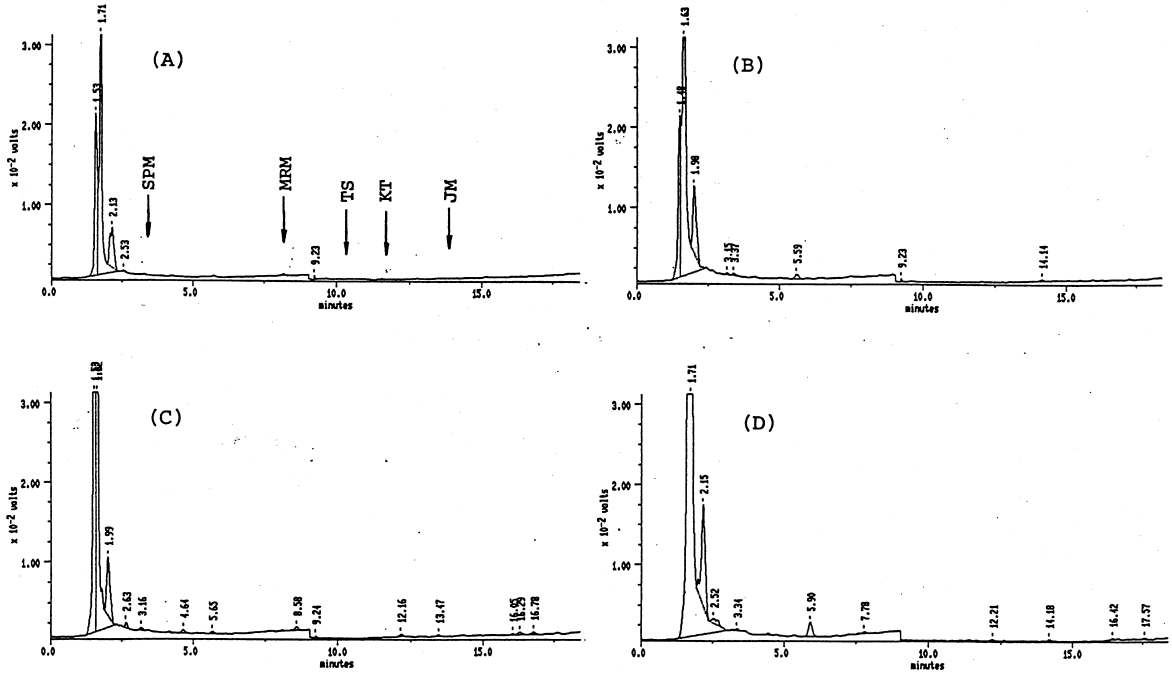

Fig. 2. Typical chromatograms obtained from commercially available meats. (A) Blank chicken muscle sample; (B) blank chicken liver sample; (C) blank swine liver sample; and (D) blank swine kidney sample. Arrows indicate retention times of spiramycin, mirosamicin, tylosin, kitasamycin and josamycin. LC conditions same as Fig. 1.

Table 4
Recoveries of josamycin, kitasamycin, mirosamicin, spiramycin and tylosin from animal tissues

Sample	Recovery (mean±S.D., n=5) (%)				
	SPM	MRM	TS	KT	JM
Chicken muscle	84.4±2.7	86.6±2.9	71.7±4.3	82.2±3.7	82.5±3.3
Swine muscle	87.1±3.0	88.1±2.5	84.1±2.9	88.4±4.0	89.7±3.7
Cattle muscle	83.0±2.4	87.6±1.9	80.2±3.5	85.2±2.7	87.5±3.1
Chicken liver	77.1±2.6	85.3±1.8	70.8±6.7	79.0±5.7	79.7±5.3
Swine liver	76.6±3.3	89.0±1.9	70.5±6.0	76.8±5.1	77.3±5.7
Cattle liver	84.2±3.6	84.8±3.0	75.4±3.2	81.0±4.7	83.2±4.8
Swine kidney	77.9±4.2	90.4±4.3	80.0±6.5	78.0±4.5	77.0±3.9

Samples were spiked with 1.0 μg/g of each drug.

obtained with each sample. The detection limits of the method were 0.05 μg/g for the five macrolides (signal-to-noise ratio >3) in each sample.

In Japan, in the development of a method for analyzing the residues, a guideline has been given: ''detection limit, not more than 0.05 μg/g; recovery under addition of 1–2 μg/g, not less than 70%; R.S.D., not more than 10%'' [26].

3.5. Comparison of HPLC and bioassay

The microbiological assays tended to lack specificity. Therefore, these methods are unsuitable for the identification of residual antibacterials. However, the microbiological assays perform very well as a qualitative method for the screening of remaining amounts of the antibacterial substances. Therefore, the antibacterial activities of the five macrolides were examined using *Bacillus subtilis* ATCC 6633, *Bacillus cereus* var. *mycoides* ATCC 11778 and *Micrococcus luteus* ATCC 9341 as test organisms. These bacteria are frequently used to test the presence of residual antibiotics in animal tissues in Japan [6]. As shown in Table 5, the antibacterial activities of the five macrolides for the test organisms, *Micrococcus luteus* ATCC 9341, were strong, and the sensitivity of the drug detection was sufficient.

Swine muscle samples fortified with JM were then analyzed by the HPLC method and the bioassay method in which *Micrococcus luteus* ATCC 9341 was used as the test organism. The concentrations obtained with the two methods showed a linear correlation (Fig. 3). The equation of the fitted curve was $y=1.04x-0.11$ ($n=12$; $r=0.98$). Similar excellent correlations were obtained for swine muscle samples fortified with KT, MRM, SPM and TS. The HPLC method with UV detection has excellent selectivity and reproducibility. However, cross-referencing the method with a conventional bioassay method makes it possible to give more accurate analytical results.

Table 5
Antibacterial activities of macrolide antibiotics

Organism	Detection limit (μg/ml)				
	JM	KT	MRM	SPM	TS
B. subtilis ATCC 6633	2.0	0.5	0.5	5.0	1.0
M. luteus ATCC 9341	0.25	0.25	0.1	0.5	0.5
B. cereus ATCC 11778	2.0	2.0	2.0	10.0	5.0

Each drug was dissolved in 0.05 *M* phosphate buffer (pH 7.0)–acetonitrile (7:3).

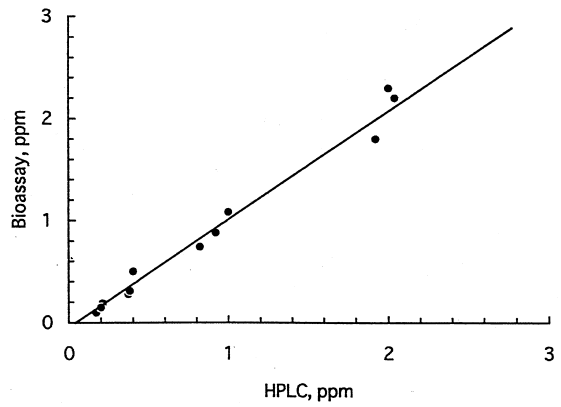

Fig. 3. Correlation between HPLC and bioassay methods for josamycin in swine muscle: $y=1.04x-0.11$; $r=0.98$; $n=12$.

4. Conclusions

The described method for the simultaneous determination of five macrolides in meat samples yielded good recoveries and precision. In addition, the detection limits of the method were 0.05 μg/g for these drugs in animal tissues, and the time required for the analysis of one sample was less than an hour and a half. Therefore, we recommend this proposed method for the routine analysis of the residual five macrolides, josamycin, kitasamycin, mirosamicin, spiramycin and tylosin, in livestock products.

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