ELSEVIER

Journal of Chromatography B, 655 (1994) 47–52

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of mirosamicin in animal tissues by high-performance liquid chromatography

Masakazu Horie[a,*], Koichi Saito[a], Norihide Nose[a], Hisao Oka[b], Hiroyuki Nakazawa[c]

[a]*Saitama Prefectural Institute of Public Health, 639-1, Kamiokubo, Urawa, Saitama 338, Japan*
[b]*Aichi Prefectural Institute of Public Health, 7-6 Tuji-machi, Kita-ku, Nagoya 462, Japan*
[c]*Department of Pharmaceutical Sciences, National Institute of Public Health 4-6-1, Shirokanedai, Minato-ku, Tokyo 108, Japan*

(First received November 4th, 1993; revised manuscript received January 25th, 1994)

Abstract

A simple and rapid method using high-performance liquid chromatography (HPLC) for the determination of mirosamicin in animal tissues has been developed. The drug was extracted with 0.3% metaphosphoric acid–methanol (7:3, v/v), and the extracts were cleaned 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 0.05 *M* phosphate buffer (pH 2.5)–acetonitrile (70:30) as the mobile phase at a flow-rate of 0.5 ml/min; the drug was detected at 230 nm with 0.04 AUFS. The calibration graph was linear from 5 to 100 ng. The recoveries of mirosamicin from various animal tissues fortified at 1.0 μg/g were 83.7–88.6% with a relative standard deviation (R.S.D.) of 2.0–5.7%. The detection limit was 0.05 μg/g.

1. Introduction

Macrolide antibiotics are a very important class of antibacterial compounds widely used in medical and veterinary practice. Mirosamicin (MRM) is one of the family of sixteen-membered ring macrolides (Fig. 1). MRM (mycinamicin II), the principal component of the mycinamycins (the mycinamycins were found to consist of five components), was isolated from the culture broth of *Micromonospora* in 1978 [1]. MRM is highly active against a wide range of Gram-positive bacteria and certain Gram-negative bacteria and mycoplasmas. MRM is absorbed well after oral administration and is distributed extensively in tissues, especially the lungs [1,2]. These characteristics make MRM suitable for the therapy of a wide range of infections. MRM has been used exclusively in livestock farming since 1988 in Japan. As a

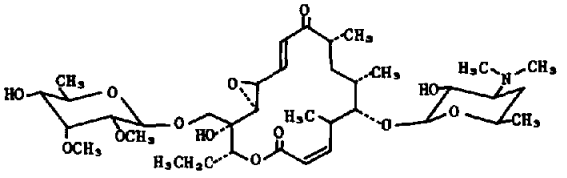

Fig. 1. Chemical structure of mirosamicin.

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00052-7

result, livestock products have become contaminated with MRM through oral administration. The presence of drug residues in livestock products is undesirable from the standpoint of food sanitation. Therefore, a simple and reliable method is needed to monitor MRM residues in animal tissues. Generally, the determination of antibiotics, including MRM, is carried out by microbiological assays. Microbiological assays are useful for the qualitative determination of remaining amounts of antibacterial substances. However, the assays tend to lack specificity, and they have difficulty in confirming the type of drug that remains 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 [3–7]. However, there are no methods available for the determination of MRM by HPLC. This paper describes a simple, rapid and reliable HPLC method for the determination of MRM in chicken, swine and cattle tissues. The technique is based on separation by reversed-phase chromatography and sample preparation with Bond Elut SCX cartridges used in a cleanup step.

2. Experimental

2.1. Materials and reagents

Mirosamycin was kindly supplied by Asahi Chemical Industry (Tokyo, Japan). 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 with 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.

2.2. Preparation of standard solutions

Stock standard solution of MRM was prepared by dissolving 50 mg of the standard preparations in 50 ml of methanol. Working standard solutions were prepared by diluting the stock solution with 0.05 *M* sodium dihydrogenphosphate (pH 4.5)–acetonitrile (7:3). The standard solutions were stored in the refrigerator in amber glass vessels.

2.3. Apparatus

The HPLC system consisted of a Shimadzu LC-6A solvent-delivery system (Kyoto, Japan), equipped with a Rheodyne 7125 injector (Berkeley, CA, USA), a Model SPD-6A spectrophotometric detector (Shimadzu) operated at 230 nm (range set at 0.04 AUFS) and a Chromatopak C-R3A data system (Shimadzu). The separation was performed on a Puresil $5C_{18}$ column (5 μm, 150 × 4.6 mm I.D., Nihon Millipore, Tokyo, Japan) with 0.05 *M* phosphate buffer (pH 2.5)–acetonitrile (70:30, v/v) as the mobile phase at a flow-rate of 0.5 ml/min. The phosphate buffer solution was filtered through a Millipore GS 0.22-μm filter (Milford, MA, USA). The chromatograph was operated at ambient temperature.

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

2.4. Sample preparation

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 gentle 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 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 onto the HPLC system.

2.5. Calibration graph

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

2.6. Microbiological assay

The antibacterial activities of MRM were measured using a paper disk method with *Micrococcus luteus* ATCC 9341 as the test organisms. The assay procedure was carried out according to the official method provided by the Ministry of Health and Welfare, Japan [8].

3. Results and discussion

3.1. Chromatographic conditions

MRM was dissolved in HPLC mobile phase and its UV spectrum was measured. The maximum UV absorption of MRM was found to be at 218 nm (Fig. 2). In the HPLC methods using UV detection of antibacterial agents it is highly important to select an appropriate detection wavelength, without any interference from contaminants. The maximum absorption wavelength of the compound to be analyzed is generally employed as the detection wavelength. In the case of a muscle sample, MRM can be detected at a high sensitivity by measuring at 218 nm. In

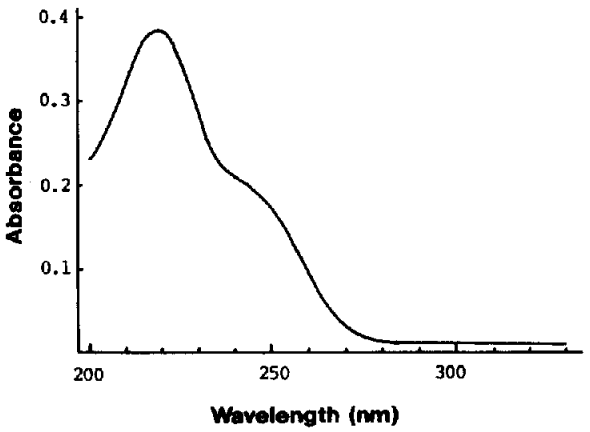

Fig. 2. Absorption spectrum of 10 μg/ml mirosamicin in the mobile phase.

the case of a liver sample, however, a large peak appeared at the solvent front, though it contained no contaminant directly interfering with the detection of MRM. Thus the measurement wavelength was set at 230 nm considering the influence of the impurity peaks at the solvent front, and with regard to a simultaneous determination with other macrolide antibiotics (for example, spiramycin and josamycin) to be developed in future. In addition, the decrease in sensitivity when MRM was measured at 230 nm was comparatively small.

Similar to a number of other macrolide antibiotics, MRM, which is a basic compound having an amino sugar in its molecule, is strongly affected by silanol groups remaining in column packing material. Therefore, the HPLC column used was a Puresil $5C_{18}$ column, an end-capped ODS column based on pure silica gel.

The choice of adequate conditions for a HPLC procedure is governed by the ionizable group, *i.e.* amino sugar, of MRM. Thus, the effects of pH of the mobile phase on peak shape and retention time of MRM were studied. The asymmetry and retention time of the peak increased with increasing pH in the range of 2.0–4.5. As a result, 0.05 *M* phosphate buffer (pH 2.5)–acetonitrile (70:30, v/v) was chosen as the mobile phase.

Generally, macrolide antibiotics are not stable

in acidic solutions. In acidic solution (pH < 4.0), almost all macrolide antibiotics are converted to degradation products. Thus, in this study, the stability of 5.0 μg/ml MRM in acidic solution (pH 2.0) at 37°C was examined by HPLC and bioassay techniques. As a result, it was found that MRM is extremely stable in acidic solution. Consequently, the use of an acidic HPLC mobile phase did not cause a problem.

Sixteen-membered ring macrolide antibiotics are mostly produced as a complex mixture of related components. As mentioned above, MRM (mycinamicin II) is one component of mycinamicins [1]. The mycinamicins were found to consist of five components. The results of the HPLC analysis of the MRM preparations under the conditions as defined in the present study indicate that, although minor components were present, the amounts of these minor components were *ca.* 10% at the most (Table 1). Therefore, it is considered that the residue characteristics can be fully evaluated by monitoring only the main component, mirosamicin (MRM).

3.2. Cleanup

The extract from a tissue sample contains many other compounds in addition to possible traces of the target compounds being tested for. It is necessary to exclude these physically- or chemically-interfering substances, and there are a variety of techniques that can be employed to accomplish this. Solid-phase extraction methods have been extensively used for cleanup of biological material prior to quantitative analysis of trace amounts of contaminants in the matrices. Skinner and Kanfer [9] used a C_{18} (ODS) cartridge to clean up josamycin form serum. Thus, we examined whether the C_{18} cartridge could be used to clean up MRM form animal tissues. Similar to other macrolide antibiotics, MRM is a highly lipophilic compound. Accordingly, it was strongly held by the C_{18} cartridge. In the case of liver and kidney samples containing many contaminants (for example, proteins, lipids and amino acids) compared with serum, however, the contaminants could not be completely eliminated by cleaning up with the C_{18} cartridge. To clean up spiramycin and tylosin contained in meat, we employed liquid–liquid extraction [5,6]. Similarly, Nagata and Saeki [4] employed liquid–liquid extraction for the cleanup of spiramycin in meat. Therefore, we discussed a cleanup procedure using liquid–liquid extraction with dichloromethane. However, this method was unsuitable for the cleanup of liver and kidney samples, because extensive emulsion formation occurred. Thus we considered another cleanup method using a cartridge packed with a silica-based cation-exchange phase. Since MRM is a basic and lipophilic compound, Bond Elut SCX packed with an ion-exchange phase (benzenesulfonylpropyl) having cationic and lipophilic properties was selected as the cartridge. To elute MRM from the cartridge, MRM was first neutralized by washing the cartridge with 0.1 *M* dipotassium hydrogenphosphate (pH 8.9) and then eluted with methanol. Using this method, test solutions free from any contaminants could be prepared (Fig. 3).

Under such analytical HPLC conditions, no interference from the antibacterial substances (other macrolide antibiotics (josamycin, kitasamycin, spiramycin and tylosin), penicillines (ampicillin, cloxacillin, dicloxacillin, nafcillin and penicillin G), tetracyclines (chlortetracycline, oxytetracycline, tetracycline and doxycycline), sulphonamides (sulphamonomethoxine, sulphadimethoxine, sulphadimidine, sulphamerazine and sulphaquinoxaline), difurazon and chloramphenicol), all having UV absorption in the vicinity of 230 nm, was observed. Only furazolidone (FZ) eluted near MRM. The re-

Table 1
Compositions of mirosamicin preparations

Sample No.	Composition (%)	
	Mirosamicin	Others
1	90.1	9.9
2	88.7	11.3
3	89.5	10.5
Reference[a]	>99.0	<1.0

Average of three trials.
[a]Reference standard preparations.

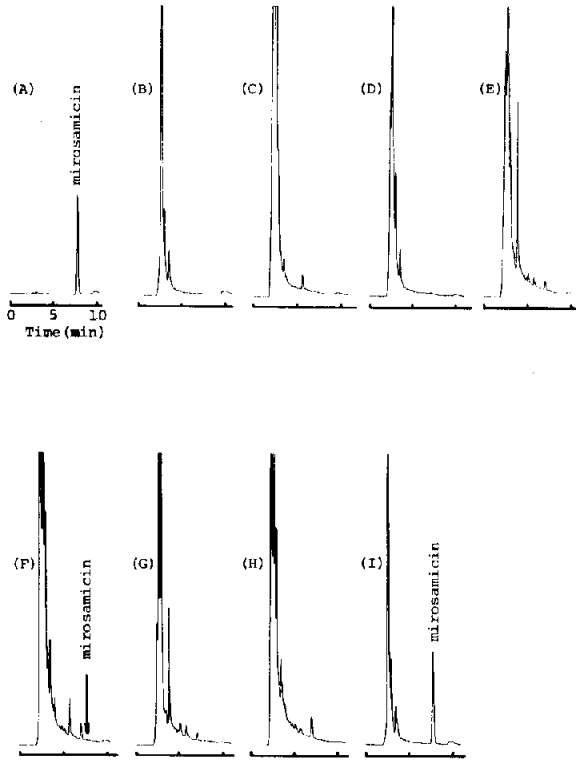

Fig. 3. Typical chromatograms of (A) standard mirosamicin (50 ng), (B) chicken muscle extract, (C) swine muscle extract, (D) cattle muscle extract, (E) chicken liver extract, (F) swine liver extract, (G) cattle liver extract, (H) swine kidney extract and (I) extract of chicken muscle fortified at 1.0 μg/g.

tention times for MRM and FZ were 7.9 and 7.3 min, respectively. However, FZ was not recovered at all by the proposed procedure.

3.3. Recovery

A linear calibration graph was obtained from 5 to 100 ng (equivalent to 0.1–2.0 μg/g) for MRM. Table 2 summarizes the recoveries of the drug 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 80% overall mean recoveries and *ca.* 5% relative standard deviations were obtained for each sample. The detection limits of MRM were 0.05 μg/g (signal-to-noise ratio >3) in each sample.

In Japan, a guideline has been given for the development of methods used to analyse antibacterial compounds; for example, detection limit: not more than 0.05 μg/g; recovery under addition of 1–2 μg/g: not less than 70%; relative standard deviation (R.S.D.): not more than 10%. Obviously, in countries having a standard residual level (for example, the USA), the detection limit should be lower than the standard residual level.

Table 2
Recoveries of mirosamicin from animal tissues

Sample	Added (μg/g)	Recovery (%)
Chicken muscle	1.0	87.1 ± 3.0
Chicken liver	1.0	85.9 ± 3.6
Swine muscle	1.0	87.3 ± 3.8
Swine liver	1.0	83.7 ± 3.5
Swine kidney	1.0	85.4 ± 5.7
Cattle muscle	1.0	88.6 ± 2.1
Cattle liver	1.0	83.9 ± 2.0

Values are mean ± R.S.D. ($n = 5$).

3.4. Comparison of HPLC and bioasasy

The microbiological assays tended to lack specificity. Therefore, such methods are unsuitable for the identification of residual antibacterials. However, microbiological assays are perfectly suitable for the qualitative determination of remaining amounts of antibacterial substances.

Chicken muscle samples fortified with MRM were then analyzed by the HPLC method and by a bioassay in which *Micrococcus luteus* ATCC 9341 was used as the test organism. This test organism is frequently used for the determination of residual macrolide antibiotics in animal tissues. The concentrations obtained with the two methods showed a linear correlation, although the quantitative data showed some differences (Fig. 4). The quantitative values were not corrected for recovery rate. The equation of the fitted curve was $y = 0.82x + 0.39$ ($n = 10$; $r = 0.97$). The HPLC method with UV detection shows an excellent selectivity and reproducibility. However, cross-reference with the conven-

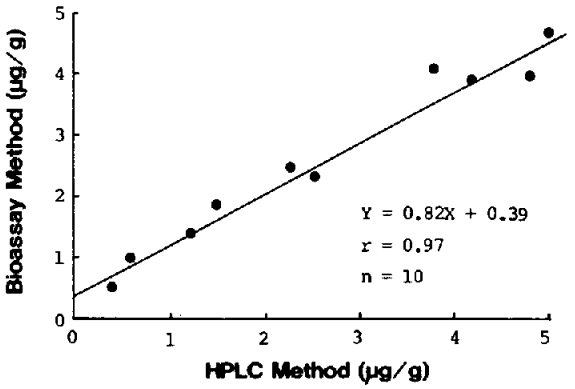

Fig. 4. Correlation between HPLC and bioassay methods for mirosamicin in chicken muscle.

tional bioassay method makes it possible to give more accurate analytical results.

The detection limits of the method were 0.05 μg/g for MRM in animal tissues, and the time required for the analysis of one sample was less than one hour. Therefore, we recommend this proposed method for the routine analysis of residual MRM in livestock products.

4. Acknowledgement

The authors would like to thank Asahi Chemical Industry for the supply of mirosamicin standard preparations.

5. References

[1] S. Satoi, N. Muto, M. Hayashi, T. Fujii and M. Otani, *J. Antibiot.*, 33 (1980) 364.
[2] N. Watanabe, *Proc. Jap. Soc. Antimicrob. Anim.*, 12 (1991) 29.
[3] W.A. Moats, E.W. Harris and N.C. Steele, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 413.
[4] T. Nagata and M. Saeki, *J. Assoc. Off. Anal. Chem.*, 69 (1986) 644.
[5] M. Horie, K. Saito, Y. Hoshino, N. Nose and H. Nakazawa, *Bunseki-kagaku*, 35 (1986) 219.
[6] M. Horie, K. Saito, Y. Hoshino, N. Nose and H. Nakazawa, *Jap. J. Toxicol. Environ. Health*, 34 (1988) 128.
[7] L.J. King and J.O. Boison, *J. Liq. Chromatogr.*, 15 (1992) 2025.
[8] Official Analytical Methods for Residual Substances in Livestock Products, Veterinary Sanitation Division, Ministry of Health and Welfare, Tokyo, Vol. 1, No. 5, 1982.
[9] M. Skinner and I. Kanfer, *J. Chromatogr.*, 459 (1988) 261.