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Determination of Josamycin Residues in Porcine Tissues Using High-performance Liquid Chromatography With Pre-column Derivatization and Spectrofluorimetric Detection*

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A simple, selective and sensitive high-performance liquid chromatographic (HPLC) method has been developed for the measurement of josamycin residues in four porcine tissues (*i.e.*, muscle, liver, kidney and fat). The sample preparation consisted of a homogenization step in an acetonitrile–10 mmol l^{-1} phosphate buffer mixture, pH 6.0 (35 + 65), centrifugation and a liquid–liquid extractive clean-up of the resulting supernatant with isooctane. Pre-column derivatization of josamycin was performed using cyclohexa-1,3-dione in ammonium acetate buffer, pH 5.0 (90 °C for 2 h). The derivative was chromatographed in an isocratic reversed-phase HPLC system. A LiChrospher RP 18 end-capped (5 μm) column was eluted with an acetonitrile–methanol–10 mmol l^{-1} phosphate buffer mixture, pH 6.0 (45 + 5 + 50). The capacity factor of the josamycin derivative was 17.5. Detection was achieved using spectrofluorimetry (λ_{ex} = 375 nm; λ_{em} = 450 nm). The structure of the derivative was assessed by using mass spectrometry. Full selectivity was obtained in the HPLC system *versus* other macrolide antibiotics (tylosin, spiramycin and erythromycin), aldehydes (formaldehyde, acetaldehyde and benzaldehyde) and endogenous compounds. Linearity and repeatability were tested. Correlation coefficients, for calibration curves in the range of 0.1–3.2 μg g^{-1}, were greater than 0.999 for all tissues and the relative standard deviation (s_r) was 4.9% (1.6 μg g^{-1}; n = 6); recovery was higher than 88%.

Keywords: Josamycin; residue; macrolide antibiotic; animal tissue; high-performance liquid chromatography; pre-column derivatization

Introduction

The use of josamycin, a macrolide antibiotic, in veterinary therapy may present a risk to the consumer, such as allergic reactions and the induction of resistant bacteria. Therefore, effective analytical methods to determine the maximum residue limit (MRL) of this drug in animal tissues have to be developed. Previously reported high-performance liquid chromatography (HPLC) techniques for the measurement of josamycin[1–4] and related macrolide antibiotics[5–8] in biological matrices have relied upon reversed-phase chromatography with ultraviolet (UV) detection. They included sample treatment either with liquid–liquid or liquid–solid extraction. There is only one report of a pre-column derivatization, using dansylhydrazine as a fluorogenic reagent.[4] These previously described methods were applied to plasma and urine samples, and assays of tissue samples concerned with other macrolides, especially tylosin.[5,7,9–11] Such work involved microbiological,[9,10] HPLC[5,7] and gas chromatography–mass spectrometry (GS–MS)[11] techniques. Although microbiological assays are suitable for screening antibiotic residues in tissue, they often lack sensitivity and selectivity and, therefore, there is a growing interest in the development of separative techniques.

At the present time, no MRLs have been published by the European Union (EU) for josamycin in meat. In order to evaluate the limit of quantitation required for the present method, limits of tolerance for related macrolides can be used as guidelines (*e.g.*, official US and Canadian tolerance values of 0.2 μg g^{-1} for tylosin;[5,7] 0.05 to 0.3 μg g^{-1} for spiramycin and 0.1 to 0.5 μg g^{-1} for tylosin in milk and different tissues from various species) for provisional MRLs in the EU[12].

We focused on the development of an HPLC system devoted to the measurement of josamycin in four different porcine tissues (*i.e.*, muscle, liver, kidney and fat) with a limit of quantitation of 0.1 μg g^{-1} and full selectivity with regard to endogenous compounds.

Experimental

Reagents

All chemicals and solvents were of analytical-reagent grade and were used without further purification. Cyclohexa-1,3-dione (CHD) was purchased from Merck-Clevenot (Nogent-sur-Marne, France); josamycin (potency equivalent to 16553 nkat mg^{-1}), tylosin, spiramycin and erythromycin were obtained from Virbac SA Laboratories (Carros, France). A 1 mg ml^{-1} solution of josamycin was prepared in methanol–water (5 + 95) and stored at 5 °C, for up to one month. Further dilutions prepared in water, to give concentrations of 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 μg ml^{-1}, were used to spike the porcine tissues tested (muscle, liver, kidney and fat).

The derivatization reagent was prepared as follows: CHD (10 mg ml^{-1}) and ammonium acetate (250 mg ml^{-1}) were dissolved in about 60 ml of water and 8 ml of concentrated HCl; the mixture was transferred into a 100 ml calibrated flask and diluted to volume with water. The reagent was kept at 5 °C and was stable for up to one month.

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Apparatus

The HPLC system consisted of a double reciprocal piston pump fitted with a membrane pulse damper (Model Spectroflow 400, Applied Biosystems, Foster City, CA, USA), an autosampler equipped with a 100 µl sample loop and a column oven (Model 507, Beckman, San Ramon, CAS, USA). Detection was operated either with a UV spectrophotometric detector (Model UV-2000, Thermo Separation Products, Les Ulis, France) or with a fluorescence detector (Model RF-551, Shimadzu, Touzart et Matignon, France), equipped with a 150-W xenon short-arc lamp. All data collection and calculations were performed using an integrator connected to a data station (Model Chromjet and 'Winner on Windows' software, Thermo Separation Products).

The fluorescence spectra of the josamycin derivative were recorded with a spectrofluorimeter (Model Fluorolog II, Spex, Longjumeau, France) using a 1 ml quartz cuvette. Mass spectrometry ion desorption chemical ionization (DCI, positive mode) was performed on a Nermag R 10-10 mass spectrometer (Argenteuil, France) in the presence of ammonia.

Tissue Sample Treatment and Derivatization Procedure

Tissue samples were left to thaw at room temperature. After mincing, 2.5 ± 0.1 g were weighed into 50 ml Virtis glass vials, then fortified with 250 µl of the appropriate spiking solution. Thirty minutes later, a 20 ml volume of acetonitrile–10 mmol l^{-1} phosphate buffer, pH 6.0 (35 + 65) was added and the mixture was blended for 10 min using a Virtis model 45 mixer fitted with U-shaped blades. The blended tissues were transferred into a 40 ml Teflon tube and centrifuged at 8500*g* for 5 min at 5 °C. The supernatant was transferred into a 25 ml calibrated flask and the volume was adjusted to 25 ml with the acetonitrile–10 mmol l^{-1} phosphate buffer mixture. A 1.5 ml volume of the resulting solution was transferred into a 10 ml screw-capped vial and extracted with 5 ml of isooctane by mechanical shaking for 10 min. After centrifuging at 3000*g* for 5 min at 5 °C, the organic layer was discarded and 0.5 ml of the derivatization reagent was added. After mixing, heating at 90 °C for 2 h and cooling, a 100 µl volume was injected into the chromatograph.

Chromatography and Detection Conditions

A guard column (4 × 4 mm i.d.) and an analytical column (125 × 4 mm i.d.) both pre-packed with LiChrospher 100 RP-18 end-capped (5 µm, Merck–Clevenot) were used.

The mobile phase for the elution of the josamycin was either acetonitrile–10 mmol l^{-1} phosphate buffer, pH 6.0 (55 + 45) or acetonitrile–10 mmol l^{-1} phosphate buffer, pH 3.5 (50 + 50). To the latter was added 5 mmol l^{-1} sodium octylsulfate. The flow rate was 1.2 ml min^{-1} and UV detection was operated at 232 nm in both instances. The mobile phase for the elution of the josamycin derivative contained acetonitrile–methanol–10 mmol l^{-1} phosphate buffer, pH 6.0 (45 + 5 + 50). The flow rate was 1.5 ml min^{-1} and the column temperature was 45 °C. Fluorescence detection was operated at an excitation wavelength of 375 nm and at an emission wavelength of 450 nm. The selected sensitivity was 'high' and the gain was set to 4.

Macroscale Synthesis, Purification and Mass Spectrometry of Josamycin Derivative

CHD (270 mg, 2.4 mmol) and ammonium acetate (930 mg, 12 mmol) were dissolved in doubly-distilled water (5 ml). The pH was adjusted to 5.0 with concentrated hydrochloric acid and the resulting solution was heated at 60 °C for 1 h. Josamycin (100 mg, 0.12 mmol) in ethanol was added to the reagent solution prepared above. After heating at 90 °C for 2 h, the solution was cooled in an ice-bath. Sodium hydrogencarbonate was added until the pH value was close to 7.0 and the solution was extracted with chloroform, three times. The organic layers were collected, dried over magnesium sulfate and evaporated to dryness under vacuum. The residue was dissolved in diethyl ether and refrigerated. A pale yellow precipitate appeared and was isolated. This operation was repeated once. A 10 mg amount of the resulting crude product was purified on a semi-preparative HPLC system: column, Hyperprep HS-BDS (C_{18} 250 × 10 mm i.d., particle size, 8 µm, from Shandon, Cergy-Pontoise, France); mobile phase, acetonitrile–water (60 + 40) at a flow rate of 4 ml min^{-1}; loop volume, 1 ml; and fluorescence detection conditions were the same as those used in the analytical system. The fraction corresponding to the main peak (retention time = 20 min) was collected, evaporated to dryness under vacuum and used for further structural and spectroscopic studies.

Results and Discussion

Guidelines to the Selection of the HPLC Assay

Previously reported HPLC techniques for the measurement of tylosin,[5,7] a macrolide antibiotic related to josamycin, in edible tissues rely upon direct UV spectrophotometric detection as josamycin has a maximum UV absorbance at a wavelength of 232 nm (molar absorbance: about 29000 l mol^{-1} cm^{-1}). However, selectivity with regard to endogenous compounds had to be improved by sample pre-treatments involving liquid–liquid and liquid–solid extraction steps. Initially, we adopted the following approach: we tested two different elution modes on the same stationary phase (*i.e.*, LiChrospher 100 RP 18 end-capped) in conditions similar to those previously described (a reversed-phase mode[3] and an ion pairing reversed-phase mode.[1] The resulting capacity factor of the josamycin peak was the same in both systems (about 6.5) and the quantitation of standard josamycin solutions was possible at a concentration as low as 0.1 µg ml^{-1} with good repeatability (the relative stand and deviation, s_r, was 1.5%, n = 6). However, UV detection at 232 nm is well-known to be prone to endogenous interferences from tissue matrices. Moreover, excellent recoveries and no dilution factor during sample preparation was necessary to reach the same limit of quantitation in tissues. To overcome these limitations, two different approaches (previously described) were tested: liquid–liquid extraction with methylene chloride[7] and liquid–solid extraction using bare silica[7] or C_{18} silica cartridges.[3] Good recoveries were obtained from standard solutions with the same quantitation limit as mentioned above. However, when these techniques were applied to tissues, interfering peaks of irregular intensity between samples were observed. Trials to combine a liquid–liquid and a liquid–solid extractive step did not give the expected selectivity whatever the chromatographic mode used. Therefore, it was decided to test a derivatization procedure involving the aldehyde group of josamycin, with an improvement in selectivity as the main criterion of reagent choice.

Development and Optimization of the HPLC Assay

Usual reagents for pre-column derivatization of aldehydes and ketones in HPLC systems are 2,4-dinitrophenylhydrazine[13] and dansylhydrazine,[4] respectively, devoted to UV spectrophotometric and spectrofluorimetric detection. More recently, a new hydrazine reagent has been introduced to give a higher fluorescent derivative,[14] *i.e.*, luminarin 3. For

amycin samycin, derivatization with dansylhydrazine has been eagent; ported[4] but this assay still required the liquid–liquid h, the ctraction of josamycin from the biological matrix. Therefore, lrogen- ıe use of specific fluorogenic reagents of the aldehyde group 7.0 and ems more advisable. Applications in HPLC mainly concern s. The actions of aliphatic aldehyde compounds with acetylace- sulfate me,[15] cyclohexa-1,3-dione (CHD)[16,17] and 5,5-dimethylcy- ıe was ohexa-1,3-dione[18] (also called 'dimedone'). yellow CHD and dimedone give higher fluorescent derivatives n was ıan acetylacetone (at least 10 to 100 times) and CHD reacts roduct ıster than dimedone.[16] For these reasons, CHD was selected lumn, ı our study in a pre-column mode. In the presence of size, 8 mmonia, CHD reacts with the aldehyde group of josamycin hase, ı give a heterocyclic structure (2, 3, 4, 5, 6, 7, 9, 10 ; loop ctahydroacridine-1,8-dione) having fluorescence properties re the Fig. 1). Reaction conditions (pH value, CHD and an excess action f ammonium) were those already optimized.[16] The reaction ı) was ate was studied as a function of the temperature and the ed for eating time. Maximum formation of the derivative was bserved with incubation at 90 °C for 2 h (Fig. 2).

The fluorescence properties and structure of the derivative ere established after its macro-scale synthesis, followed by urification in a semi-preparative HPLC system, without uffer salt in the mobile phase, to facilitate further spectroscopic studies. The isolated eluting fraction corresponding to he main peak was used to record the fluorescence (Fig. 3) and he mass spectra (Fig. 4). Excitation and emission wavelength alues were 375 and 450 nm respectively; the mass fragmentation pattern gave characteristic peaks of m/z values higher han the molecular mass of josamycin, (M = 828), which orresponded to the molecular ion of the derivative (m/z = 015) and to a fragment resulting from the loss of an acetyl roup on the josamycin moiety (m/z = 956).

For analytical purposes, the derivative was chromatographed in a reversed-phase mode. Two organic modifiers acetonitrile and methanol) were used, concomitantly, in the nobile phase in order to increase the resolution between the eak of the josamycin derivative and an interfering peak from he reagent (Fig. 5). Chromatographic characteristics of the eak of the josamycin derivative are summarized in Table 1.

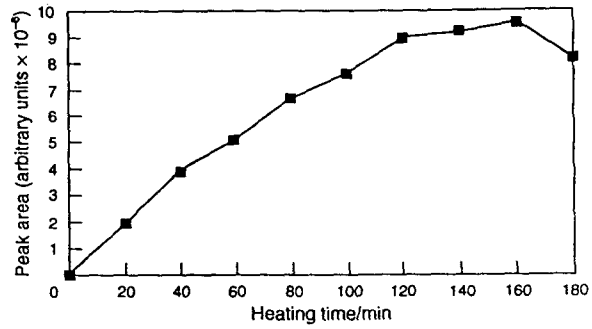

Fig. 2 Time-course for the formation of the josamycin derivative. The temperature was kept at 90 °C and the concentration of the josamycin standard solution was 0.08 μg ml^{-1}.

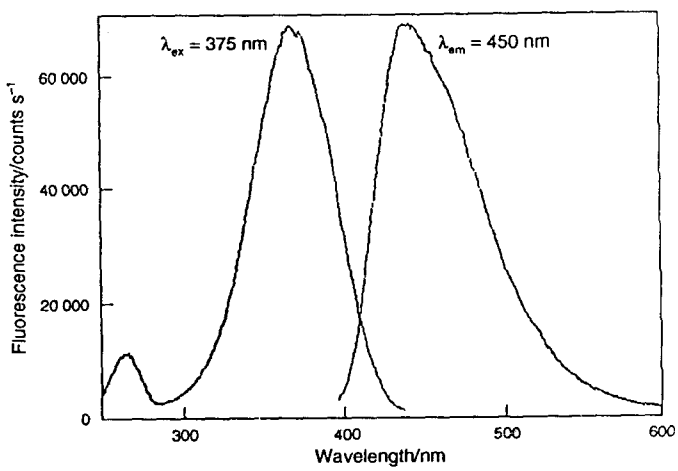

Fig. 3 Fluorescence excitation and emission spectra of the josamycin derivative measured in the mobile phase.

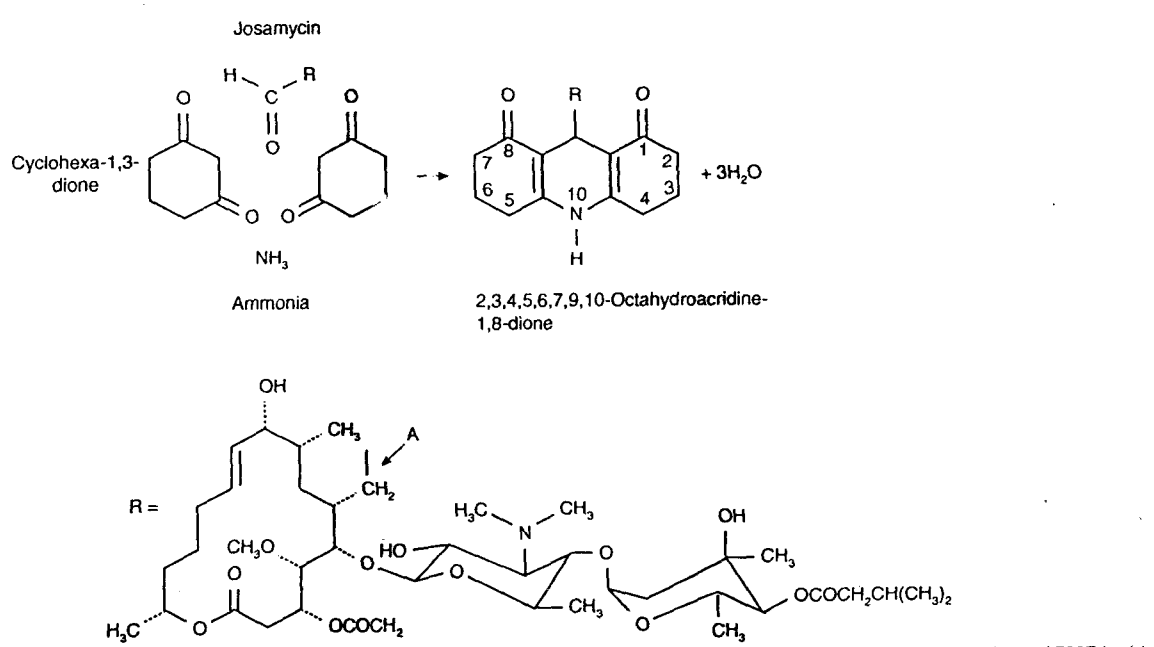

Fig. 1 Reaction scheme of the formation of the fluorescent derivative between josamycin, ammonia and cyclohexa-1,3-dione (CHD). (A indicates the methylene group of josamycin bound to the fluorophore).

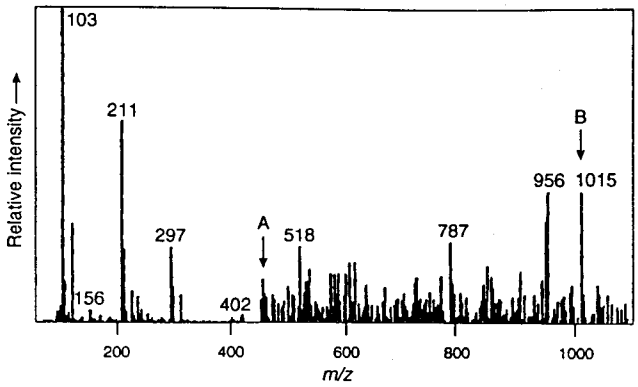

Fig. 4 Mass spectrum of the josamycin derivative obtained by macroscale synthesis and purified by semi-preparative HPLC (ion desorption chemical ionization mode in the presence of ammonia). Part of the spectrum at A (after m/z = 450 is expanded 50 times). (B indicates the molecular ion of the josamycin derivative: m/z = 1015).

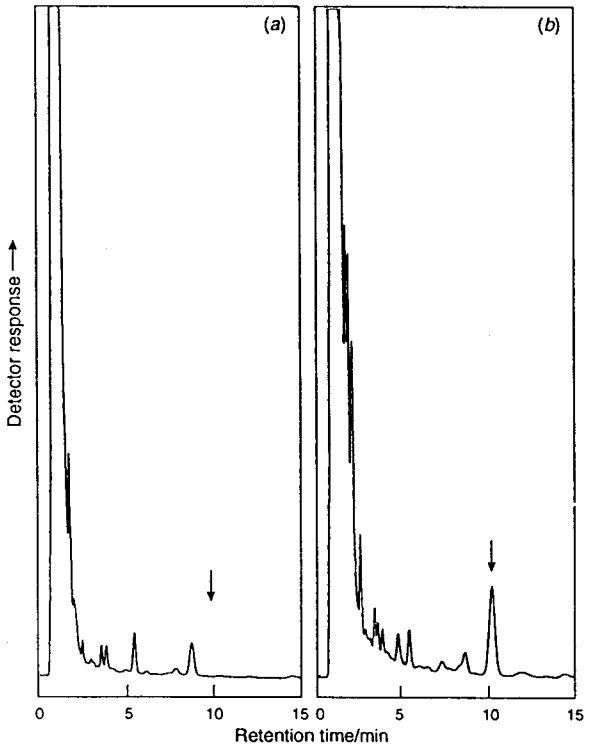

Fig. 5 Typical chromatograms corresponding to (*a*) a reagent blank and (*b*) a josamycin standard solution (0.08 μg ml^{-1}) derivatized in optimized conditions (90 °C and 2 h). HPLC operating conditions: LiChrospher RP18 end-capped (5 μm) 125 × 4 mm i.d. eluted with acetonitrile–methanol–10 mmol l^{-1} phosphate buffer, pH 6.0 (45 + 5 + 50) at a flow rate of 1.5 ml min^{-1} and a column temperature of 45 °C; spectrofluorimetric detection (λ_{ex} = 375 nm, λ_{em} = 450 nm).

Table 1 Chromatographic parameters of the josamycin derivative peak obtained in the analytical HPLC system

Retention time/min	10.3
Capacity factor	17.5
Number of theoretical plates m^{-1}	27 700
Tailing factor	1.14
Selectivity*	1.17
Resolution*	2.50

* Calculated *versus* adjacent reagent peak.

Before derivatization, treatment of tissues included different steps. Homogenization in a phosphate buffer, containing acetonitrile, precipitates the proteins. A 35% (v/v) acetonitrile content seemed well-fitted to the sample handling, as a lower value implied protein precipitation during heating and a higher value resulted in foam formation leading to difficulties in collection of the extractive layer. After homogenization, liquid–liquid extractive clean-up step with isooctane had to be performed to eliminate an endogenous interfering peak.

Assay Validation

The developed HPLC assay was selective against by-products of the reaction (Fig. 5), other usual aldehyde compounds (formaldehyde, acetaldehyde and benzaldehyde derivatives were eluted earlier than the josamycin derivative) and related macrolide antibiotics (*i.e.*, erythromycin, spiramycin and tylosin). Erythromycin gave no peak, as expected, as it has no aldehyde group in its structure; spiramycin and tylosin gave main peaks with a relative retention time *versus* josamycin of 0.27 and 0.36, respectively. Full selectivity was also obtained with regard to endogenous compounds of the different porcine tissues studied. Fig. 6 shows typical chromatograms obtained for a blank and for muscle, kidney, liver and fat samples spiked at a concentration of 0.8 μg g^{-1}.

Linearity of the method was tested for concentrations of josamycin ranging from 0.1 to 3.2 μg g^{-1}. Equations for regression lines, correlation coefficients and recoveries (spiking level: 0.8 μg g^{-1}, n = 6) are reported in Table 2. The s_r values, calculated for repeatability, (n = 6) were 8.9 and 4.9% at 0.2 and 1.6 μg g^{-1}, respectively. The limit of detection (signal-to-noise ratio = 3) was about 25 ng g^{-1} in each tissue tested.

The present method appears to be valuable for josamycin residue analysis. It could be adapted to other aldehyde

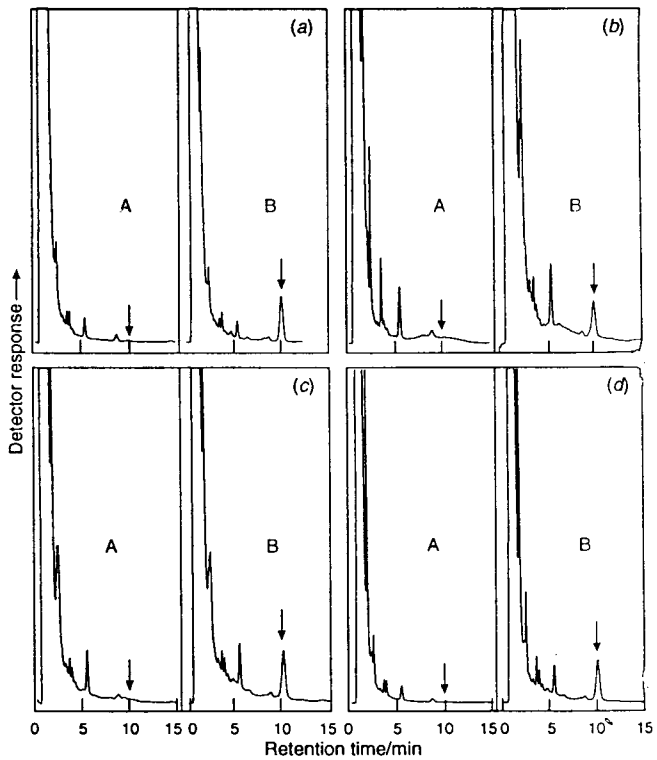

Fig. 6 Typical chromatographic profiles obtained with tissue samples: (*a*) muscle, (*b*) liver, (*c*) kidney, and (*d*) fat. A, Blank, and B, fortified with 0.8 μg g^{-1} of josamycin. HPLC operating conditions were the same as in Fig. 5.

Table 2 Validation parameters for josamycin assay in spiked porcine tissues

	Linearity		Recovery*
Tissue	Equation of the regression line†	Correlation coefficient	(% ± s_r) ($n = 6$)
Muscle	$y = 8\,555\,845\,x - 102\,614$	0.9997	92.1 ± 4.5
Liver	$y = 8\,425\,450\,x - 249\,473$	0.9993	88.1 ± 4.3
Kidney	$y = 7\,053\,252\,x + 595\,865$	0.9992	91.9 ± 4.5
Fat	$y = 8\,444\,552\,x + 116\,115$	0.9996	90.6 ± 4.4

* Tested at a concentration of 0.8 μg g^{-1}.
† Where x is the josamycin concentration (0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 μg g^{-1}) and y is the peak area (arbitrary units).

macrolide antibiotics, provided that minor changes in sample treatment and mobile phase composition were introduced.

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