



## Study of the metabolism of spiramycin in pig liver

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Received 23 June 1997; received in revised form 23 September 1997; accepted 23 September 1997

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### Abstract

A major metabolic pathway of spiramycins in pig liver is described. This biochemical reaction involves L-cysteine – a common amino acid present in most animal tissues – which reacts with the aldehyde function of the antibiotic forming a thiazolidine ring. This transformation of spiramycin derivatives drastically increased their polarity. A preliminary HPLC method enabling the quantitation of each metabolite in the range 0.5 µg/g of liver tissue is proposed. Spiramycin S is used as an internal standard while extraction procedures take into account the physico-chemical properties of the thiazolidine moieties. By comparison, previous HPLC methods underestimated the exact amount of antibiotic residues because these metabolites were not extracted from the studied tissues. © 1997 Elsevier Science B.V.

**Keywords:** Spiramycin

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### 1. Introduction

Spiramycin is a mixture of three macrolide antibiotics mainly constituted of spiramycin I (over 85%) while spiramycins II and III are minor derivatives (percentages respectively lower than 5 and 10%) (Fig. 1).

Due to its absence of toxicity at therapeutic doses both in man and animals, spiramycin has been extensively used to overcome various bacterial infections (mainly respiratory disorders). For many years, the characterization and quantitation of metabolites remained a subject of discussion. First in 1968, Terlain and Jolles [1] reported the presence of a polar conjugate of neospiramycin revealed thanks to the wide range of solvents used during extraction

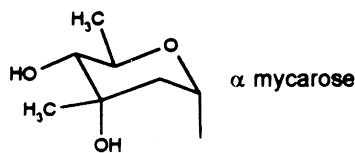
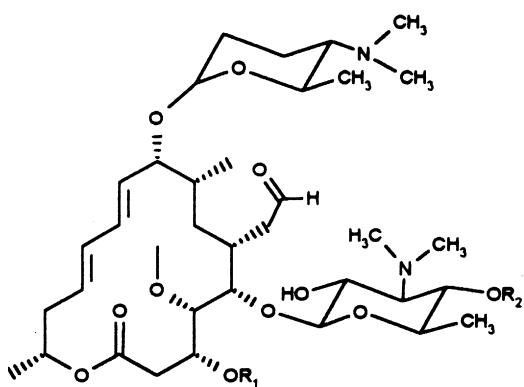
procedures. Unfortunately, the structure of this metabolite remained unknown. Later on, many studies were devoted to the pharmacokinetics of spiramycin I and neospiramycin I [2–8] or to the assay of spiramycin I [9–14] but no progress occurred on identification of spiramycin metabolites. The main problem with these studies was their extraction procedure using chlorinated solvents. Even though it has been shown to be valid for spiramycin I and neospiramycin I, it was inefficient to extract the polar metabolites.

The instability of spiramycin I in liver or kidney has been mentioned [2,3], but additives such as  $\text{CoCl}_2$  [2–4] avoiding the degradation of spiked spiramycin seemed sufficient to prevent this biochemical phenomenon.

In the present work, a study of the stability of spiramycin in pig liver was conducted. It clearly revealed the biotransformation of spiramycins into

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$R_1 = H, R_2 = \alpha$  mycarose : Spiramycin I

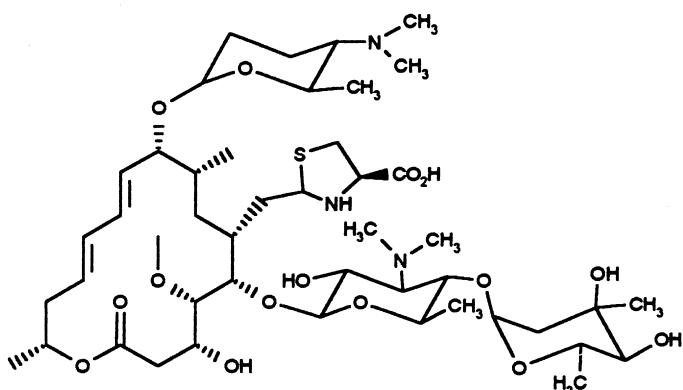
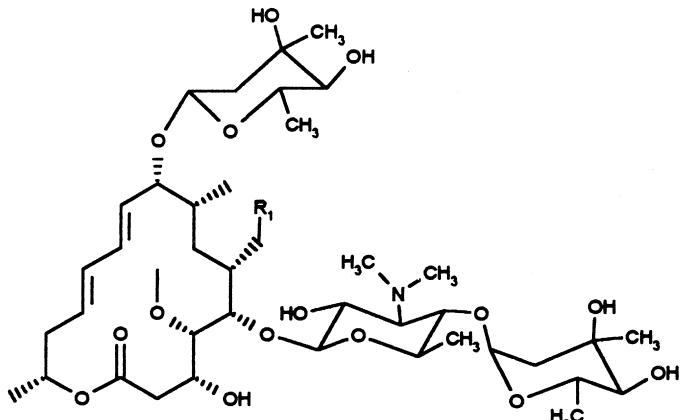
$R_1 = COCH_3, R_2 = \alpha$  mycarose : Spiramycin II

$R_1 = COCH_2CH_3, R_2 = \alpha$  mycarose : Spiramycin III

$R_1 = H, R_2 = H$  : Neospiramycin I

$R_1 = COCH_3, R_2 = H$  : Neospiramycin II

$R_1 = COCH_2CH_3, R_2 = H$  : Neospiramycin III



$R_1 = COH$  : Spiramycin U  
 $R_1 = CH_2OH$  : Spiramycin S

### Cysteyl Spiramycin I

Fig. 1. Structures of spiramycins, neospiramycins, spiramycins U and S and cysteyl spiramycin.

polar cysteyl derivatives. These metabolites were found to constitute about 80% of the residues. As a consequence, a simple quantitation of spiramycin I

and neospiramycin I in a cysteine rich tissue such as liver underestimated the effective quantity of spiramycin residues by a factor between 10 and 5.

## 2. Experimental

### 2.1. Animals

The study of metabolism was conducted on the liver of a treated piglet provided for 7 days ad libitum with a feed containing 400 µg/g of spiramycin embonate. It was slaughtered 12 h after its last feed.

### 2.2. Materials and reagents

All reagents were of analytical grade. Acetonitrile, methanol and chloroform were obtained from Prolabo (Paris, France) and were of HPLC-quality grade. Water was purified through a Milli-Q system (Millipore, Bedford, MA, USA).

Neospiramycin I, the demycarosyl residue of spiramycin I was easily obtained from spiramycin I in acidic media (pH<2).

Authentic cysteyl derivatives were prepared by addition to spiramycin of an excess of L-cysteine base in a 0.02 M  $\text{Na}_2\text{HPO}_4$ – $\text{CH}_3\text{OH}$  (70–30 v/v) medium.

Spiramycin U is a natural impurity of spiramycin. Spiramycin S was synthesized by reduction of spiramycin U by  $\text{NaBH}_4$  in methanol, and used as internal standard.

### 2.3. Extraction procedure

Extraction protocol used at the beginning of our study of spiramycin metabolism, so called ‘alkaline methanol’ extraction method, was a simplified version of an extraction method used for microbiological assays. A 3 g aliquot was sampled and added to 30 ml of methanol–0.1 M sodium phosphate buffer pH 9, (70:30, v/v). The mixture was then stirred for 30 min and then submitted to 10 min of centrifugation (3500 rpm). The supernatant was separated and diluted twice with water before HPLC injection. This method was assumed to be quantitative for the extraction of spiramycin residues, but it also extracted other impurities from pig liver, thus hindering the UV detection, especially when trace level was reached.

This is why another method was developed in order to enhance the selectivity toward spiramycin

residues: a 5 g aliquot of ground pig liver was added to 5 ml of water and magnetically stirred for 5 min, resulting in a very fine dispersion of the sample. Next, 40 ml of acetonitrile, mixed with 5 ml of a 25 mg/l of spiramycin S in acetonitrile, used here as internal standard, were gradually added to the aqueous suspension of the pig liver with continuous magnetic stirring. After 15 min, the mixture was centrifuged at 3500 rpm. Five to 20 ml of the supernatant phase were submitted for 10 min under 200 mm Hg bench vacuum to evaporate the acetonitrile. A 1.5 ml volume of methanol was then added to the aqueous residue, and the total volume adjusted to 5 ml by water addition. The mixture was homogenized in an ultrasonic bath and filtered through 0.2 µm nylon filters. A 2 ml aliquot of the filtered mixture was injected into the precolumn of the HPLC system.

### 2.4. Structural analysis

NMR experiments were performed in  $\text{DMSO-D}_6$ , using a 400 MHz AM400 Bruker Spectrometer. Assignment of all the resonances was obtained thanks to 2D homo- and heteronuclear experiments (COSY 45 and HMQC). Mass spectrometry was performed using a Sciex API III spectrometer in the electrospray mode.

### 2.5. Instrumentation and chromatographic method

The HPLC system consisted of 3 Gilson Model 305 pumps (Villiers-le-Bel, France). Column temperature was controlled by circulating water through a jacket using a thermo electric heating/cooling accessory. The column switching system was obtained from Touzart and Matignon (Les Ulis, France). Detection was carried out with a Hewlett Packard (Palo Alto, CA, USA) 1040 diode array detector (DAD).

A basic HPLC system (HPLC Method 1), used at the beginning of our study, was operated isocratically at room temperature using a reversed-phase Nucleosil C<sub>8</sub> column (250×4.6 mm I.D.; 5 µm particle size) (Touzart and Matignon, Les Ulis, France). Mobile phase was a mixture of acetonitrile–0.05 M sodium phosphate buffer pH 2.2, (65:35, v/v) to which 6 g/l of  $\text{NaClO}_4$  were added. The

flow-rate was 1 ml/min. Twenty to 50  $\mu$ l were injected through a conventional Rheodyne injection loop. Multi-wavelength detection at 200 nm, 232 nm and 280 nm was achieved with a 1040 DAD.

Later, the HPLC system had to be adapted to the analysis of the transformation products of spiramycin (HPLC Method 2, Fig. 2). A preconcentration step through a precolumn (Fig. 2) was added to reach trace levels present in animal tissues. The HPLC system was operated isocratically at 60°C to avoid peak splitting due to the interconversion of thiazolidine isomers [21]. This system included 2 columns: a reversed-phase Nucleosil C<sub>8</sub> column (30×4.6 mm I.D.; 5  $\mu$ m particle size) (Touzart and Matignon, Les Ulis, France) was used for sample clean up and preconcentration step; a Kromasil C<sub>8</sub> column (250×4.6 mm I.D.; 5  $\mu$ m particle size) (Touzart and Matignon, Les Ulis, France) was used for analytical separations.

The precolumn was initially out of the column circuit, and equilibrated with a relatively non-eluting solvent through Pump 2. This 'concentration mobile phase' was a 0.05 M sodium phosphate buffer pH 2.3–CH<sub>3</sub>CN (94:6, v/v) NaClO<sub>4</sub> 6 g/l. The flow-rate was 1.3 ml/min.

Once the precolumn was conditioned (about 10 min), up to 2 ml of extract were injected. The maximum percentage of methanol in the sample was 40% (v/v) in order to trap all spiramycin derivatives and especially the most polar one, i.e. those transformed by cysteine.

Two min after the injection, the precolumn was switched in series with the column for 2 min. The mobile phase used for the separation was a mixture of 0.05 M sodium phosphate buffer pH 2.3–CH<sub>3</sub>CN (67:33, v/v); NaClO<sub>4</sub> 6 g/l. The flow-rate was 1.1 ml/min.

It was not necessary to leave the precolumn in series with the analytical one longer than 2 min. In that way, the most tightly retained hydrophobic solutes were not able to elute, thus contaminating subsequent analyses due to their late elution time. So, while separation was occurring, the precolumn was switched off 2 min after injection and acetonitrile was circulated through it for 5 min using Pump 3. The precolumn was then reconditioned with the concentration solvent (Pump 2) while the analysis still went on (Fig. 2).

Approximate retention times obtained are listed in Table 1. The close time found for cysteyl spiramycin I and neospiramycin I could explain why these compounds were previously confused. Multi-wavelength detection was identical to that used in HPLC Method 1.

The molar response coefficient of all spiramycin derivatives were taken equal to that of spiramycin base, which is rational since the only UV absorbing function of spiramycin, i.e. the two conjugated double bonds of the macrolide, was not implied in

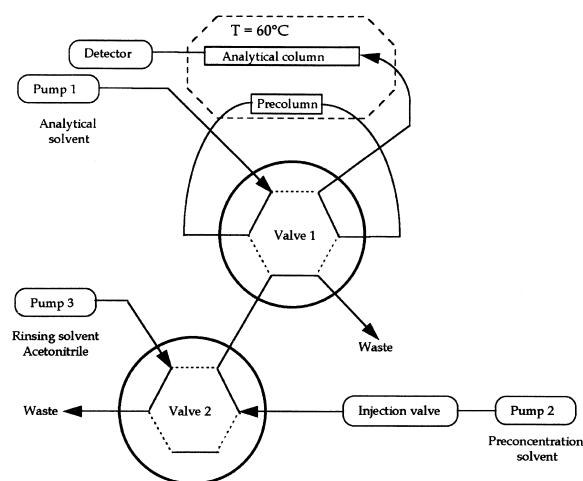


Fig. 2. Three-pump chromatographic system used for spiramycin analysis in pig liver. Valve positions are the following (arrows indicate the flow of the eluents):  $t=0$  min: injection; Valve 1 (---), Valve 2 (—);  $t=2$  min: Valve 1 (—), Valve 2 (—);  $t=4$  min: Valve 1 (---), Valve 2 (—);  $t=9$  min: precolumn rinse, Valve 1 (---), Valve 2 (---);  $t=20$  min: precolumn conditioning, Valve 1 (---), Valve 2 (—).

Table 1  
HPLC retention times of spiramycin derivatives using the pre-column concentration system at 60°C (HPLC Method 2)

Solute	Retention time (min)
Cysteyl neospiramycin I	4.34
Cysteyl spiramycin I	5.2
Neospiramycin I	5.7
Spiramycin I	7.75
Cysteyl spiramycin U	7.8
Cysteyl spiramycin III	10.1
Spiramycin S	13.2
Spiramycin III	15.5
Spiramycin U	16.8

the transformation. The quantity of each metabolite was first expressed in quantity equivalent of spiramycin S, the internal standard. The response coefficient of spiramycin S being taken equal to that of spiramycin I, the content of all metabolites were expressed in equivalent quantity of spiramycin I.

### 3. Results and discussion

#### 3.1. Transformation of the spiramycin derivatives

The metabolism of the antibiotic by the liver was studied extensively. Spiramycin derivatives were added to pig liver, to determine which reactions occurred. Several derivatives of spiramycin, such as spiramycin I and III, neospiramycin I or spiramycin U were quickly transformed. This transformation can be followed on the chromatograms of the spiked extracts by the formation of peaks having identical UV spectrum but eluting at lower retention times, which is characteristic of the formation of derivatives with higher polarity (Fig. 3).

The only compounds that remained unchanged were the derivatives of spiramycin with a reduced aldehyde group (either  $\text{CH}_2\text{OH}$  or  $\text{CH}_3$ ) such as spiramycin S. This demonstrated the implication of the aldehyde function of the macrocycle in the reaction. It was also observed that the reaction was partially reversible (between 5 and 10%) in acidic media or when samples were kept a long time in the refrigerator.

When the derivatives of spiramycin were added at 1000  $\mu\text{g/g}$  in the liver, the transformed compound was present in sufficiently high quantity to be in part extracted by chloroform enabling their structural determination using mass spectrometry. The observed molecular weight corresponded to that of the initial derivatives, increased by 103 Da. If the hypothesis of the aldehyde reaction was admitted, with the loss of  $\text{H}_2\text{O}$ , it was consistent to consider the addition of a molecule of mass  $103+18=121$ , which considerably reduced the possibilities. In fact, L-cysteine was a very likely candidate.

As the reaction of spiramycin with L-cysteine can be easily obtained in low alkaline ( $\text{pH}>7$ ) media, a

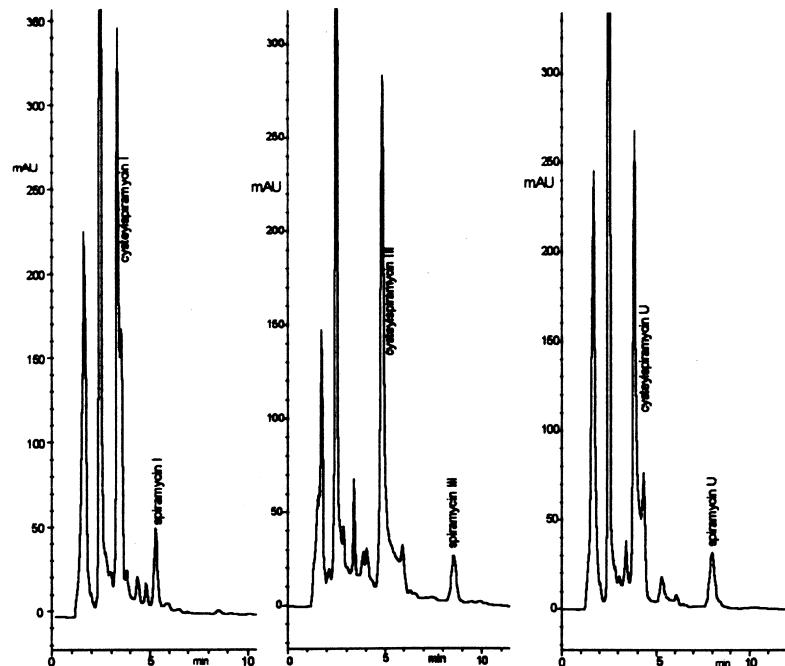


Fig. 3. Chromatograms of 'alkaline methanol' liver extracts with a starting adduct of 200  $\mu\text{g/g}$  of spiramycin I, of spiramycin III, and spiramycin U. Detection: 232 nm; HPLC Method 1.

complete structural determination of the synthesized derivative led to the structure shown in Fig. 1. Fig. 4 shows the obtained mass spectrum.

2D NMR experiments (both homo and heteronuclear) were performed at 400 MHz in DMSO D<sub>6</sub>. The thiazolidine resonances of the 2 isomers (50/50) were assigned at: <sup>1</sup>H,  $\delta$ =4.8 and 4.6 ppm (dd, thioaminal protons), 3.9 and 3.6 ppm (dd,  $\alpha$  protons) and 2.8 and 3.1 ppm (dd,  $\beta$  protons); while the respective <sup>13</sup>C resonances were determined at  $\delta$ =69 and 70 ppm (thioaminal carbons), 65 and 67 ppm ( $\alpha$  methine carbons) and 30 and 31 ( $\beta$  methylene). The chemical shifts pertaining to the other protons were similar to those of the parent compound in the range of  $\pm 0.15$  ppm.

The reaction of aldehydes with L-cysteine is well known and has been extensively described for formaldehyde [15–19]. It involves several consecutive chemical equilibria whose kinetics depend on physico-chemical parameters such as: pH, ionic

strength, presence of buffer and temperature. Particularly, the pH and the cysteine concentration are of utmost importance. Similar trends are observed with spiramycin. The reaction is slow in acidic media but is faster at increasing pH (about 6).

Complete structural analysis was not conducted on the spiramycin adducts obtained after incubation with liver. Nevertheless, the mass spectra of these spiramycin metabolites were identical to those obtained from synthetic work. In addition, all the chromatographic profiles of these compounds were respectively superimposable.

The acidity constants of cysteal derivatives remained unchanged concerning the two N(CH<sub>3</sub>)<sub>2</sub> showing a pK<sub>a</sub> close to 7.7 while the pK<sub>a</sub> of the carboxylic function is 1.5. The polar character of the molecule makes it difficult to be extracted in chlorinated solvents (dichloromethane, chloroform). This could be explained by the presence of the carboxylic acid function, fully ionised in alkaline media. The

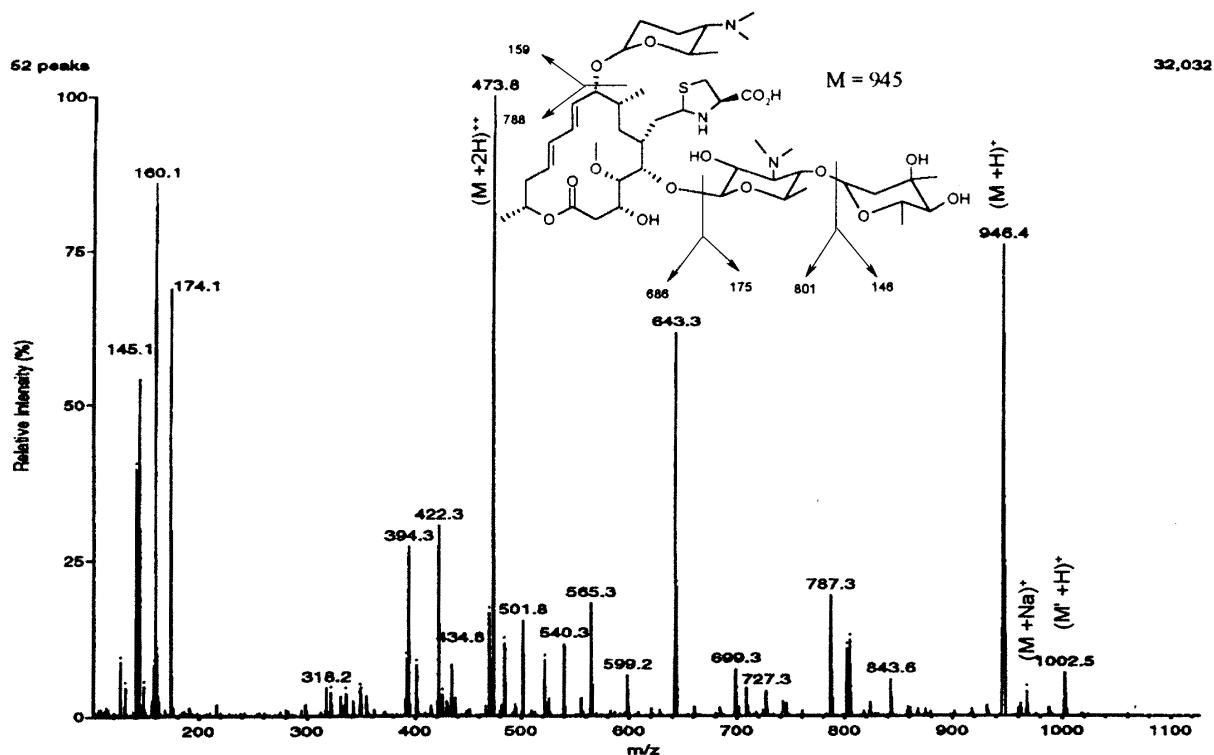


Fig. 4. Electrospray mass spectrum (TIC) of cysteal spiramycin I ( $M'=1001$  is due to traces of cysteal spiramycin III).

$K_a$  of the NH function of the thiazolidine group was much lower than that of amino acids in general, and of cysteine in particular, since it should be about 6.3 [15,17].

It is important to point out that the reverse reaction where cysteyl derivatives are partially transformed back to the starting material may occur in the absence of cysteine and in acidic media. However, this reaction is rather slow and minor. Studies [18] on 2-substituted aliphatic thiazolidine-4-carboxylic acid showed that decomposition was mainly obtained in strong basic solutions (1–3 M NaOH). This degradation work was not conducted on spiramycin due to the opening of the macrocycle in these conditions.

Sanders [2–4] reported that the extensive use of additional  $\text{CoCl}_2$  inhibited 'spiramycin degradation' in liver. In fact, the complexation of  $\text{CoCl}_2$  with L-cysteine [22–24] avoided any further reaction of the spiked free spiramycin used as standard. On the other hand the cysteyl spiramycin compounds remained unchanged because of the very slow kinetic rate of the back transformation. The extracting solvent used ( $\text{CHCl}_3$ ) was too apolar to efficiently extract all the cysteyl derivatives. As a matter of fact, the partition coefficient  $K = C_{\text{org}}/C_{\text{aq}}$  of the cysteyl derivatives in the system  $\text{CHCl}_3/\text{buffer pH 9}$  is between 1.5 and 2. Consequently the given recovery rates, calculated on the spiked spiramycin, were not representative of the overall spiramycin residues effectively present in liver tissue.

Reactivity of other compounds chemically close to cysteine were also tested. It leads to the following conclusions: when the cysteamine group is present in the reactant molecule (ethyl or methyl ester of L-cysteine), the reaction occurred. On the other hand, if the thiol function is protected no reaction could be observed (homocysteine, cystine or methionine). Glutathione and serine gave no reaction.

### 3.2. Extraction methods

The solvent used should lead to the complete extraction of both spiramycin and their cysteyl derivatives. It should thus have a solubilizing capacity over a sufficiently broad polarity range.

The extracting efficiency of previously published methods – valid for spiramycin I and neospiramycin

I – were first examined for cysteyl derivatives. The one set up by Sanders et al. [2–4] consisted of a chloroformic extraction from various animal tissues such as liver, muscle or kidneys, added with an aqueous  $\text{K}_2\text{HPO}_4$  solution in presence of  $\text{CoCl}_2$ . After centrifugation, a triphasic system was obtained: chloroformic and aqueous phases were present with an intermediate phase constituted of an emulsion of liver with chloroform. A sample of blank pig liver has been first spiked with a mixture of spiramycin I and spiramycin S, both at 25  $\mu\text{g/g}$  and then extracted following the described procedure. Spiramycin I, cysteyl spiramycin I and spiramycin S were determined in each phase. The liver residue was further consecutively extracted three more times with methanol to check the extraction efficiency. We found after summing the contribution of each phase that 70–80% of the spiked spiramycin I was transformed into cysteyl spiramycin I. The bottom chloroformic phase contained 10% of the spiramycin residue: 6% due to the free spiramycin I but only 4% of cysteyl spiramycin I. The remaining 90% were present in the top aqueous phase and in the liver emulsion. This assay seemed to show that this type of method extracted about 8% of cysteyl derivatives and should result in an underestimation of the residue by a mean factor of 5–10, knowing that the obtained value was not reproducible due to volume variations of chloroform still emulsified with liver.

As a consequence, extraction with pure methanol was considered, because it both extracted spiramycin and its cysteyl metabolites. However, it also extracted other biomolecules interfering with the metabolites. Extractions with pure acetonitrile were unsuccessful, since liver tissues tended to agglomerate in this medium. Only 40% of the cysteyl conjugates were then extracted.

The property of water to disperse the liver tissue was used to develop extraction conditions with acetonitrile–water (90:10) (10 ml per g of liver). A good dispersion of the liver was then obtained and pollution by polar interfering compounds coextracted from the liver was limited. Attention should be paid because evaporation of acetonitrile led to precipitation while the subsequent addition of methanol was not sufficient to dissolve all the solid material. That is why the mixture had to be filtered through 0.2  $\mu\text{m}$  nylon filters.

### 3.3. HPLC method

Column temperature appeared to have a key role on the chromatographic profiles of the cysteinyl conjugates. At room temperature, they had a split shape as shown in Fig. 3. When column temperature was decreased under 10°C, 2 isomers peaks were obtained for each cysteinyl derivative. Over 50°C, the 2 peaks coalesced. This phenomenon, well known in chromatography, is due to the simultaneous presence of two interconverting compounds [20]. Their interconverting rate is very sensitive to temperature. The resulting peak shape depends on the limiting step between the interconversion of the diastereoisomers and the chromatographic mass transfers. The opening of a thiazolidine ring via a probable imino intermediate resulting into an equilibrium between two diastereoisomers has been already described for many 2-substituted thiazolidine carboxylic acid derivatives [21].

### 3.4. Study in liver of treated pig

The chromatogram of the extract shows four chromatographic peaks with a spiramycin like UV spectrum (Fig. 5). The presence of cysteinyl neospiramycin I was demonstrated by controlled additions. This derivative can be easily synthesized either by reaction of neospiramycin I with cysteine, or by acid degradation (pH 2) of cysteinyl spiramycin I.

Significant quantities of cysteinyl spiramycin III could also be identified in the extract. Free spiramycin I and III were both present but in very small quantities.

The peak of cysteinyl neospiramycin III, eluting at about 8 min, is very close to that of spiramycin I. In the chromatogram of the extract (Fig. 5a), the peak thus obtained was rather broad and could be attributed to the presence of a mixture of spiramycin I and cysteinyl neospiramycin III. An excess of L-cysteine was added to the extract (Fig. 5b) in order to try to completely transform spiramycin into its metabolites (10 min at pH 8.6 in a  $\text{Na}_2\text{HPO}_4$  buffer). Then the peak due to spiramycin III is strongly lowered proving the relative efficiency of the reaction. Consequently the remaining peak at 8 min was mainly due to cysteinyl neospiramycin III.

Each form of spiramycin (I or III) is transformed

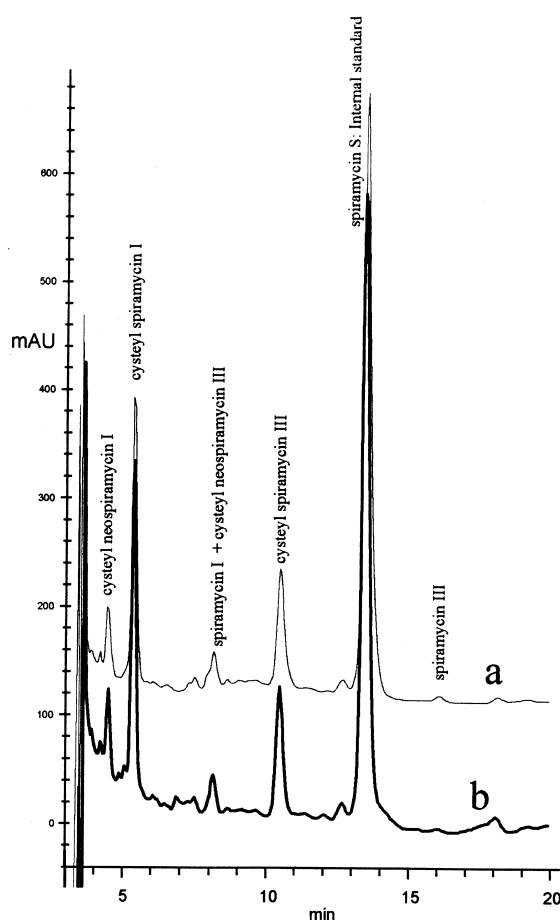


Fig. 5. Chromatogram of an extract from a treated pig liver (extraction: water– $\text{CH}_3\text{CN}$  (10:90), detection 232 nm; HPLC Method 2); (a) liver extract; (b) liver extract with adduct of L-cysteine.

in the liver extracts under three compounds: cysteinyl spiramycin, cysteinyl neospiramycin and spiramycin base. We can thus propose that the metabolism of spiramycin is mainly governed by two chemical reactions: cyclization with cysteine and hydrolysis of mycarose giving neospiramycin derivatives. This later transformation is logical when low pH value of gastric medium is considered.

Analytical results are gathered on Table 2. Precise limit of quantitation was not measured but since the interfering peaks due to the liver must be taken into account, it was difficult to reach quantities lower than 0.25  $\mu\text{g/g}$ .

An unexpected result has to be pointed out. While

Table 2  
Determination of spiramycin derivatives using HPLC Method 2

	Cysteyl neospira I	Cysteyl spira I	Spira I	Cysteyl neospira III	Cysteyl spira III	Internal standard	Spira III
Peak areas	510	2815	100	450	1820	9500	100
Content (µg/g)	1.2	6.4	<0.25	1.0	4.1	21.5	<0.25

the ratio of spiramycin I/spiramycin III observed in the regular spiramycin produced by Rhône Poulen Rorer is typically in the range 15–25, the ratio of the respective metabolites obtained in peak liver is 1.5, i.e. almost 10 times lower than what could be expected. As the biotransformation of spiramycin I into spiramycin III has to be discarded, it means that faster elimination occurred for spiramycin I derivative. On the other hand, as cysteyl conjugates are probably the main elimination route of the starting material, one could guess that differences in reactivity towards cysteine also play a major role in this observation. Additional work should be done to raise definitive conclusions.

#### 4. Conclusions

As a conclusion, this work clarifies the metabolism of spiramycins in vivo. It shows that about 80% of spiramycin in pig liver is transformed by L-cysteine into a thiazolidine carboxylic acid derivative. The relative amount of cysteyl adducts depends on the concentration of cysteine in the studied tissue. Since the polarity of these derivatives is drastically higher than that of free spiramycin derivatives, their extraction by conventional chlorinated solvent was not completely achieved. As a consequence, previous extraction methods were not adequate and led to a large underestimation of spiramycin residues at least in pig liver.

As cysteine is a common amino-acid, these conclusions should be extended to all the animals species. Other studies involving rabbit tissues are under investigations.

#### Acknowledgements

Dr. J.M. Paris, Dr. J.C. Barrière and Dr. D. Deprez are warmly acknowledged for helpful discussions.

We thank the Structural Analysis Department for structural determinations.

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