DETERMINATION OF SPIRAMYCIN AND NEOSPIRAMYCIN RESIDUES IN BOVINE FAT TISSUE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.

G. Casademont, J. Vilageliu*, E. Castells ° and J.A. García Regueiro.
IRTA. Unitat Química Alimentària. Centre de Tecnologia de la Carn. 17121 Monells.Girona. (Spain)
*** CIDA s.a.l Centro Industrial Santiga. C/.Argenters, 6. 08130 Sta. Perpètua de Mogoda (Barcelona)**
° J.Uriach & Cia S.A. C/.Degà Bahí, 59-67. 08026 Barcelona

Background

Spiramycin is a macrolide antibiotic used in veterinary practice. EC Regulations set maximum residue limits (MRL) of drugs in different animal products and tissues. Compliance with such Regulations requires sensitive analytical techniques for residues analysis. MRL for spiramycin and its metabolite neospiramycin, is 300 mg/kg in bovine fat tissue (1). For this reason there is a need to develop an analytical procedure for determination of spiramycin and neospiramycin in this matrix.
Microbiological tests and enzyme immunoassays have been used for determination of several antibiotic residues, including spiramycin, in milk and eggs. A study of seven different microbial inhibitor tests for detection of antibiotics in milk concluded that no test was suitable for detection of spiramycin (2). TLC/bioautobiographic analysis of macrolide antibiotics in poultry meat and milk (3) and tissues, milk and eggs (4) were also reported. HPLC procedures have been described for quantification of spiramycin in chicken muscle (5), but few methods have been reported for determination of both spiramycin and neospiramycin. A RP-HPLC method was successfully applied in muscle, kidney and liver (6,7) and in plasma and milk (8). Recently, a particle beam/liquid chromatographic/mass spectrometric method for confirmatory analysis of spiramycin (9) and for simultaneous determination of five macrolides (10) in muscle have been reported. However, up to this point, there is a lack of studies for the determination of spiramycin and neospiramycin in fat tissue.

Objective

The main aim of this study was to develop an analytical method that would be suitable for quantitative determination of residual spiramycin and its metabolite neospiramycin in bovine fat tissue.

Method

Reagents

HPLC grade acetonitrile were used to prepare the mobile phase. All other chemicals were of analytical grade or similar. SPE cartridges were Bond Elut, diol, 3cc/500mg from Varian (Harbor City, CA, USA). Spiramycin was purchased from Sigma (St. Louis, MO, USA) and Neospiramycin was obtained by acid hydrolysis of spiramycin. Stock standard solutions were prepared containing 1 mg/ml and 0,1 mg/ml in methanol. Working standards were prepared with appropriate dilution from the stock solutions. Internal standard solution was p-Nitrophenol in methanol (0.1 mg/ml). Injection solution was methanol-2% formic acid-acetonitrile (45:35:20).

Apparatus and HPLC conditions

The Liquid Chromatographic System consisted of a Waters 600 pump, a Waters U6K injector and a Waters 990 photodiode detector operated at 231 nm. The analytical column was a Supelcosil LC-8DB, 5µm, 150x4.6 mm i.d. from Supelco (Bellefonte, PA, USA). Mobile phase consisted of a 0.5 % sulphuric acid solution- acetonitrile (77:23) at a flow rate of 1.5 ml/min.

Procedure

20 ml of chloroform were added to 2 g of homogenised fat tissue. The mixture was stirred and ultrasonicated for 5 min and centrifuged over 10 min at 3500 rpm. The supernatant was filtered through a folded paper and the extraction was repeated with an additional 20 ml of chloroform, and the filter was rinsed with a 2-3 ml of chloroform. The combined extracts were evaporated in a rotary evaporator and the residue was redissolved in 2 ml chloroform. A 50 µl p-nitrophenol solution was added at this point, when it was used. A diol SPE column was preconditioned by washing with 1 ml chloroform and sample extract was passed through the cartridge, using a vacuum manifold with low vacuum. Cartridge was washed with 10 ml chloroform-hexane (5:95). After drying with vacuum (2.5 min), sample was eluted with 7 ml TEA 1% in methanol. The eluate was evaporated in a rotary evaporator and residue was transferred to a tube with two 500 µl portions methanol. Methanol was evaporated with a stream of nitrogen and samples were then reconstituted with 250 µl of injection solution. It was necessary to perform a centrifugation step before chromatographic analysis in order to obtain clean final extracts. 50 µl were injected into the HPLC system.

Results and discussion

Chromatograms showed no interference and a good resolution at any of the retention times that corresponded with those of both analytes and the internal standard. Figure 1 presents the chromatogram of an extract of fat spiked at 2.0 µg/g level. To assess linearity, a set of spiked fat samples containing 0.3, 0.5, 1.0, 2.0, 3.0 and 4.0 µg/g concentration of spiramycin and neospiramycin were prepared by adding appropriate amounts of working standard solutions. Samples were carried through the complete procedure as described above. The respective peak height ratios between spiramycin and neospiramycin and internal standard were calculated and a linear-regression curve by least-squares computation was calculated. Linear equations (r^2>0.99) were obtained over the range studied. Either peak height or the rate between peak height of analyte and internal standard could be used for calculation, but results using internal standard gave slightly better linearity.
Precision of analytical procedure was estimated by processing multiple replicates (n=4) of fortified fat samples at 0.3, 0.5 and 2.0 µg/g levels. Concentrations of analyte were calculated using analytical curves prepared by spiking fat at 6 different levels and constructed at the same day of analysis. Table 1 presents the means, standard deviations (SD) and relative standard deviations (RSD)

obtained. Precision values were measured by calculated RSD and were within an acceptable range, RSD < 15 %, and similar for both compounds.

Recovery experiments were carried out on fat tissue at spiking levels of 0.3, 0.5 and 2.0 µg/g. Samples were carried through extraction, clean-up and anlysis as described. The same day, a set of five standards containing from 0.6 to 6.0 µg of each spiramycin and neospiramycin in 250 µl of injection solution, which is equivalent to concentrations in tissue from 0.3 to 3.0 µg/g, were prepared. Direct injections of these standards (50 µl) were chromatographied and yielded linear equations. Recoveries were determinated by reading each peak heigh obtained from spiked samples in the corresponding standard curve without matrix. Results are shown in Table 2. The absolute recovery of neospiramycin was found to be between 50-60 %, while spiramycin was recovered 53-66 %. Relative standard deviations were < 15 % in all cases. The use of an internal standard, added at the beginning of the analytical procedure likely would most correct recoveries.

Conclusions

This methodology can be used to quantitative analysis of spiramycin and its metabolite neospiramycin in bovine fat tissue, in a concentration range of 0.3 to 4.0 µg/g. It shows a good precision, RSD range 7-15 %. The absolute analytical recoveries, although they are not very high (50-60 %), they present low variability, they do not exceed 15 % RSD.

Bibliographie

(1) Commision of the European Communities. Council Regulation EC 1442/95. Off. J. Eur. Commun., L143 (1995), 26.
(2) G. Suhren and W. Heeschen, Milchwissensch., 49, 11, (1994), 629.
(3) I. Schwaiger, R. Schuch and H. Hoertner, proceedings of Euro Food Chem VIII, Vienna, 3, (1995), 727.
(4) M. Petz, R. Solly, M. Lymburn and M. H. Clear. J.Assoc. Off. Anal. Chem, 70, 4, (1987), 691.
(5) T. Nagata and M. Saeki, J. Assoc. Off. Anal. chem., 69, 4, (1986), 644.
(6) P. Sanders, P. Guillot, G. Moulin, B. Delépine and D. Mourot. Proceedings Euroresidue Noordwijkerhout, (1990), 315.
(7) P. Sanders, P. Guillot, M. dagorn, G. Moulin, B. Delépine, D. Mourot. Am. J. Vet. Res., 55, 3, (1994), 358.
(8) L. Renard, P. Henry, P. Sanders, M. Laurentie and J.M. Delmas, J. Chromatogr. B, 657, (1994), 219.
(9) P. Sanders and B. Delépine. Biol. Mass Spectrom., 23, (1994), 369.
(10) B. Delépine, D. Hurtaud-Pessel and P. Sanders, J. Assoc. Off. Anal. Chem., 79, 2, (1996), 397.

TABLE 1. PRECISION OF ANALYTICAL PROCEDURE .
(n=4)

Added (ng/g)	NEOSPIRAMYCIN		SPIRAMYCIN	
	Amount founded mean ±SD (ng/g)	RSD (%)	Amount founded mean ±SD (ng/g)	RSD (%)
300	307.25±32.42	10.5	330.95±36.28	10.9
500	606.58±44.34	7.3	646.00±67.47	10.4
2000	2111.24±298.59	13.1	2147.47±322.27	15.0

TABLE 2. ANALYTICAL RECOVERY.
(n=4)

Added (ng/g)	NEOSPIRAMYCIN		SPIRAMYCIN	
	Mean recovery ±SD (%)	RSD (%)	Mean recovery ±SD (%)	RSD (%)
300	59.98±6.51	10.8	66.11±5.31	8.0
500	58.21±7.69	13.2	62.44±6.54	10.4
2000	50.59±7.18	14.2	52.62±7.84	14.9

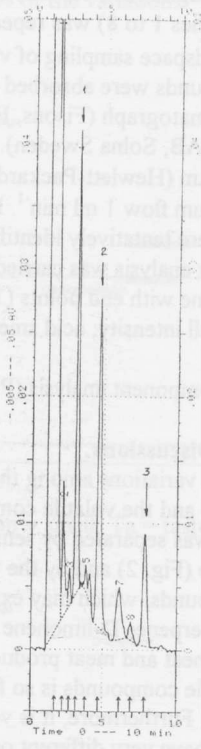

Fig.1 Chromatogram of spiked fat sample
(1) Neospiramycin
(2) Spiramycin
(3) p-Nitrophenol