

## PREVALENCE OF MOLECULES OF $\beta$ -LACTAM ANTIBIOTICS IN BOVINE MILK IN LOMBARDIA AND EMILIA ROMAGNA (ITALY)

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### Introduction

The  $\beta$ -lactams are the oldest group but still one of the most employed among the groups of antibiotics. This group bothers human health; as a matter of fact, cases of allergic reactions due to residues in milk are described in literature (Freeman T. R., 1953; Kindred T. P. and Hubbert W. T., 1993). Because of this, the molecules belonging to the group of  $\beta$ -lactams have the lowest tolerances in the EU between all the antimicrobials. The EU Regulation 2377/90 set these Maximum Residue Limit (MRL) for some  $\beta$ -lactam antibiotics in milk: penicillin G 4  $\mu\text{g/l}$ , ampicillin 4  $\mu\text{g/l}$ , oxacillin 30  $\mu\text{g/l}$ , amoxicillin 4  $\mu\text{g/l}$ , dicloxacillin 30  $\mu\text{g/l}$ , cephalixin 100  $\mu\text{g/l}$ , cephapirin 60  $\mu\text{g/l}$ .

The analysis to detect antimicrobial residues in milk are usually performed in two steps: first a microbial or enzymatic or receptor-based method is used as a screening tool. Second, the samples found positive are confirmed by a chemical method. A confirmatory method has to be able to detect which molecule is present in the sample and to quantitate it. High pressure liquid chromatography coupled with UV detector (HPLC-UV) is the technique usually adopted as a confirmatory method for antibiotic residues. This technique has some limitations: mainly it has a low sensitivity and selectivity, therefore many purification steps are needed (Faria Reyes J. F. *et al.*, 2000; Taguchi S. *et al.* 1999; Sorensen L. K. *et al.*, 1997). Sometimes, in order to detect the analytes through a fluorescence detector, also a derivatisation step can be used to achieve higher sensitivity (Edder P. *et al.*, 1999; Marchetti M. *et al.*, 2002; Berger K. and Petz M., 1991). In any case the methods are quite time consuming and unsuitable to process a great number of samples. This could be one of the reasons of the lack of data in national and international literature in terms of molecules more frequently present as residues in milk.

Moreover, the HPLC-UV confirmation, even using a diode array detector, is not completely reliable (Mitchell J. L. *et al.*, 1998). The EU Commission Decision 93/256 already stated "Methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods". Recently the commission decision 2002/657 confirmed and strengthened this concept.

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Liquid chromatography coupled with mass spectrometry (HPLC-MS) could represent a highly sensitive and selective tool to detect antibiotic residues in milk without the need of many purification steps. There are already some methods to detect residues of  $\beta$ -lactam antibiotics in milk using HPLC-MS, but in many cases they are unsatisfactory because they are unable to reach the sensitivity required by the tolerances set by the EU Regulation 2377/90. Only recently some technical improvement of the HPLC-MS technique, in particular of the interface, allowed some sensitive methods to be set up (Riediker S. and Stadler R. H., 2001; Daeseleire E. *et al.*, 2000). In order to analyse a great number of samples found positive by a screening method we developed a fast method based on HPLC-MS/MS. The method has been validated and used to analyse 53 bovine milk samples found positive by a microbial test at the milk section of the IZSLER of Brescia (Italy) during routine quality controls. During 2001 this official organisation tested 159.543 samples, 549 of which (0.34%) were found positive by the microbial screening test.

## Materials and Methods

### Sampling

53 bovine milk samples were randomly chosen between the samples that during 2001 tested positive by a microbial test (Delvotest SP<sup>®</sup>) at the milk section of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER, Brescia, Italy) during routine quality controls. The samples were collected at 4 different times during 2001. The samples were stored at  $-20^{\circ}\text{C}$  and analysed within one month from their collection.

### Extraction

5 ml of raw milk were put into 10 ml Pyrex screw cap centrifuge tubes and vortex mixed with 400  $\mu\text{l}$  of 10% acetic acid aqueous solution. The acidified milk was then centrifuged at 3500 r.p.m. for 10 minutes at  $4^{\circ}\text{C}$ . The clear supernatant phase was taken by a syringe, avoiding taking the upper fat layer, and filtered through a 0.50  $\mu\text{m}$  nylon filter 13 mm diameter (Advantec MFS, Pleasanton, USA). The filtered extract was put into 2 ml autosampler vials (Chromacol Ltd., Herts, UK) and 50  $\mu\text{l}$  were injected into the HPLC-MS/MS system. Each sample was prepared in duplicate.

### Liquid chromatography-mass spectrometry

High pressure liquid chromatography was carried out using a P2000 series binary pump (Spectra Physics, Milan, Italy) equipped with an AS3000 autosampler (Thermo Finnigan, San Jose, CA, USA) provided with a 50  $\mu\text{L}$  loop. The analytes were separated on a Merck-LiChrospher 100 RP18 (250 mm x 4 mm i.d., 5  $\mu\text{m}$ , Merck, Darmstadt, Germany) column. Water and acetonitrile both acidified with 0.1% formic acid were used as mobile phase at 1 ml/min. constant flow, according to the following elution program: from 100% water to 100% acetonitrile in 6 min. and from 100% acetonitrile to 100% water from 6 to 12 min. A 2 minutes equilibration delay was used between each chromatographic run.

The column effluent was connected to the Peek transfer line (1m x 0,25 mm) of

the mass spectrometer interface and subjected to a 70:30 split ratio. MS/MS analyses were carried out on an API 365 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo Ion Spray interface for pneumatically assisted electrospray.

The molecular and fragment ions of the investigated analytes are reported in Table 1.

Molecule	Molecular ion [M+H] <sup>+</sup>	Fragment ion	Fragment ion	Fragment ion	Fragment ion
Penicillin G	335	<b>160</b>	289	176	
Ampicillin	350	<b>106</b>	160	174	192
Oxacillin	402	<b>160</b>	243		
Amoxicillin	366	<b>114</b>	160	208	349
Nafcillin	415	<b>199</b>	300	171	256
Dicloxacillin	470	<b>160</b>	311		
Cephalexin	348	<b>158</b>	106	174	192
Cephapirin	424	<b>292</b>	181	141	

**Table 1:** The molecular ion and the fragments found for each molecule. The ions in bold are those of the most sensitive transition.

MS/MS acquisitions were performed in the mass reaction monitoring mode (MRM) by monitoring the signal of the most abundant fragment ion.

### Quantitation

Quantitation was made through external standards. Calibration curves were built injecting mixtures of stock solutions at the following concentrations: 5, 10, 50, 100, 200, 400, 1000 µg/l. Nafcillin was used as internal standard, not for quantitation, but only to check the effective recovery of the analytes. The results of the samples were corrected for the recovery value. This value was calculated for each molecule as the mean of 12 recovery experiments at two concentration levels (20 and 400 µg/l).

The limit of detection (LOD) and quantitation (LOQ) of each antibiotic, defined as the injected amount giving a signal-to-noise ratio of 3 and 10 respectively, were determined by the analysis of spiked milk samples.

The percent of recovery of the antibiotics were calculated at two concentration levels (20 and 400 µg/l) from six spiked milk samples.

## Results and discussion

### Mass spectrometry

Ito Y. *et al.* (2001) described the use of electrospray ionization tandem mass spectrometry run in negative ion mode to detect residues of β-lactam antibiotics in tissues. Negative ion mode was tried but, in our laboratory, gave sensitivities from six to ten times lower than positive ion mode.

Several authors have already described the fragmentation pattern of penicillins (Siegel M. M. *et al.*, 1987; Tyczkowska K. *et al.*, 1989; Suwanrunpha S. *et al.*, 1988; Suwanrunpha S. and Freas R. B., 1989). In any case the most important mechanism of fragmentation of these molecules is the cleavage of the β-lactam ring that gives

origin to the fragment ion at  $m/z$  160, which is class specific of penicillins, and its complementary  $m/z$   $[M+H-159]^+$  fragment which is compound-specific. Other ions are due to the loss of small lateral groups from the entire molecule or from the two described fragment ions.

The cleavage of the  $\beta$ -lactam ring can be described also for the cephalosporins. In the case of cephalexin the fragment ion at  $m/z$  158 has the same origin of the  $m/z$  160 ion found for penicillins. In this case the complementary ion is present too at  $m/z$  192. The fragment ion at  $m/z$  106 is due to the loss of the aromatic ring plus a carbon atom and the bound  $-NH_2$ . Concerning cephalixin the cleavage of  $\beta$ -lactam ring should produce two ions at  $m/z$  208 and 215 that were not present in the product ion mass spectra. As a matter of fact, the fragmentation of the molecular ion of cephalixin gave origin to three ions respectively at  $m/z$  141, 181 and 292. The first one could be produced from  $m/z$  208 losing a carbonyl group and the second one from 215 losing the lateral group  $-CH_2OCOCH_3$ . The origin of  $m/z$  292, the most abundant fragment ion, is unknown. In any case the  $m/z$  424 ion is the father of  $m/z$  292 as it has been confirmed by the precursor ion scan. Daeseleire E. *et al.* (2000) found the same ion and, being the most abundant fragment ion, used it for quantitation.

#### Analysis of the samples

Penicillin was found in 26 over the 53 analysed samples (24 at concentrations higher than the MRL and 2 lower). Amoxicillin was found only in 3 samples at concentrations higher than the MRL. Cephalixin was found in 2 samples at concentrations lower than the MRL. The results of the analytical determinations are presented in Table 2.

Only penicillin G, amoxicillin and cephalixin were detected in 29 over the 53 samples analysed. No other molecules were found and the reasons of this could be different:

The samples were false positive at the microbial test. Having false positive results is tolerable for a screening test that has to be very sensitive, but not very selective. The most important feature of screening tests is that they don't give false negative results.

Molecules different than those included in our method were present in the samples. This is possible because the number of molecules that can be used in the therapy of bovine mastitis is high. For the most recent molecules it is impossible to get the standards from commercial retailers.

The finding of penicillin G, amoxicillin and cephalixin is consistent with the broad use of these three molecules in the therapy of bovine mastitis and with the results of other studies.

Moats W. A. (1999) analysed by a confirmatory HPLC based method 54 samples found positive by  $\beta$ -lactam antibiotics screening tests. Penicillin G was detected in 20 samples, ampicillin in 3, amoxicillin in 1, cephalixin in 9, desacetylcephalixin in 2. In 12 samples no  $\beta$ -lactam antibiotics were detected. The prevalence of the molecules is a little different from that registered in this research. However, it has to be mentioned that the samples analysed by Moats had different origins. 50 samples were from different sources in the USA and 4 from Barbados.

Sample	Analyte	Concentration ( $\mu\text{g/l}$ )
12	penicillin G	6.4 $\pm$ 0.6
15	penicillin G	39.6 $\pm$ 4.1
16	amoxicillin	38.8 $\pm$ 0.1
18	penicillin G	6240 $\pm$ 450
19	penicillin G	140 $\pm$ 28
20	penicillin G	90.3 $\pm$ 4.1
21	penicillin G	155 $\pm$ 3
22	penicillin G	829 $\pm$ 9
23	penicillin G	102 $\pm$ 1
25	amoxicillin	53.7 $\pm$ 2.3
26	penicillin G	11.6 $\pm$ 1.1
27	penicillin G	101 $\pm$ 1
28	penicillin G	18.8 $\pm$ 1
31	penicillin G	750 $\pm$ 133
33	penicillin G	6.2 $\pm$ 0.6
34	penicillin G	1380 $\pm$ 100
35	penicillin G	1580 $\pm$ 148
37	penicillin G	3.7 $\pm$ 0.4
38	penicillin G	13.7 $\pm$ 0.2
39	penicillin G	13.1 $\pm$ 0.2
40	penicillin G	36.5 $\pm$ 7.7
41	penicillin G	3.8 $\pm$ 0.4
42	penicillin G	77.3 $\pm$ 1.5
43	cephapirin	6.4 $\pm$ 0.3
44	cephapirin	15.7 $\pm$ 0.1
45	penicillin G	319 $\pm$ 37
46	penicillin G	19.8 $\pm$ 2.2
48	penicillin G	212 $\pm$ 12
51	penicillin G	91.7 $\pm$ 12.7
52	penicillin G	11.9 $\pm$ 0.5
53	amoxicillin	8.5 $\pm$ 0.1

**Table 2:** Concentrations of the analytes detected in each sample (mean  $\pm$  standard deviation). The samples were prepared in double. Sample 18 was diluted 1 to 5 in order to be determined within the linear range of the calibration curve.

Riediker S. *et al.* (2001) analysed by HPLC-MS/MS 18 samples found positive by screening tests. They detected amoxicillin in 4 samples, ampicillin in 3, cloxacillin in 7 and penicillin G in 8. In some cases more than one molecule was detected in one sample. In 2 samples no  $\beta$ -lactam antibiotics were detected (false positives). Also in this research penicillin G was the most detected  $\beta$ -lactam antibiotic.

The prevalence of false positives in that research is lower than that of the present research. This is due to the fact that they used a cross control between two different screening tests (Delvotest SP<sup>®</sup> and BetaStar<sup>®</sup>). It has been shown that Delvotest SP<sup>®</sup>, used in this research, is not specific for  $\beta$ -lactam antibiotics, and it can give false positive results, especially if the somatic cells count is high (Kang J. H. and Kondo F., 2001) or after some feed supplementation (Romnee J. M. *et al.*, 1999). Delvotest SP<sup>®</sup> can become more specific for  $\beta$ -lactam antibiotics only if preceded or followed by  $\beta$  lactamase treatment. The fact that Riediker S. *et al.* had lower detection limits than those achieved by the method proposed here can explain only partially the difference.

53 is a representative sample of the population of the 549 samples found positive during 2001. We can, therefore, assume that penicillin G was present in 269 over 549 (49.1%) positive samples, amoxicillin in 31 (5.7%) and cephalirin in 21 (3.8%).

## Conclusions

A fast confirmatory method able to detect residues of seven  $\beta$ -lactam antibiotics at sub M,R,L. levels was developed using HPLC-MS/MS. The mass spectrometric behaviour of these molecules was studied. The results obtained from the analytical determination performed over the samples we had from the Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna showed that, in Northern Italy, penicillin G is the  $\beta$ -lactam antibiotic which is present more frequently in milk as a residue. Amoxicillin and cephalirin too can be found as residues in milk, but less frequently and at levels lower than penicillin. In any case the prevalence of milk samples found positive at the microbial test is very low and it doesn't pose risks for the consumers' health. Some other new  $\beta$ -lactam molecules could be included in the method, but a certain difficulty to obtain the standards from the producers has to be mentioned.

**Keywords:**  $\beta$ -lactams, antibiotics, milk, HPLC-MS

**ABSTRACT** - There is a lack of information about which molecules of antibiotics are more often present as residues in milk. An HPLC-MS/MS method was then developed and used to confirm 53 bovine raw milk samples that were found positive by a microbial method (Delvotest SP<sup>®</sup>) at the Istituto Zooprofilattico Sperimentale per la Lombardia e l'Emilia Romagna of Brescia (Italy) during 2001. Penicillin G was found in 26 samples at concentrations ranging from  $3.7 \pm 0.4$   $\mu\text{g/l}$  to  $6240 \pm 550$   $\mu\text{g/l}$ . Amoxicillin was found in 3 samples at concentrations ranging from  $8.5 \pm 0.1$   $\mu\text{g/l}$  to  $53.7 \pm 2.3$   $\mu\text{g/l}$ . Cephalirin was found in two samples at the concentration of  $5.7 \pm 0.1$   $\mu\text{g/l}$  and  $6.4 \pm 0.3$   $\mu\text{g/l}$ .

*from the Ph.D. thesis of Sergio Ghidini*

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