Residues of veterinary drugs in Slovenian milk 1995-1998

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Abstract

The control of veterinary drug residues in milk is performed in Slovenia. The monitoring of antibiotic residues in milk began in 1977. In the last four-year period (1995-1998) a total of 3358 samples were analysed and the majority of them (99.4 %) were negative. Antibiotics were found in only 19 samples. Determination of sulfonamides in milk was introduced in 1995. In four years we examined 406 samples, all of them negative. Residue monitoring was extended again in 1996 with the determination of chloramphenicol, and a total of 217 samples were analysed. One sample was confirmed to contain 4.6 μg/kg of chloramphenicol even though use of it is prohibited for food-producing animals. In 1998, 50 samples of milk were also examined for anthelmintic ivermectin; no residues were found.

Key words: milk samples, analysis, antibiotics residue

Introduction

The production of healthy milk is of great importance. During production a great number of veterinary medicinal products can be used for the prevention and cure of animal diseases, especially mastitis. Veterinary drugs can be present in milk as a consequence of treating the whole organism, but their presence after intramammary application is even more frequent. Residues of veterinary drugs can persist in milk, depending on the type of drug, even for several days. Some of the veterinary medicinal products are prohibited for the treatment of animals used for milk production for human beings. The reason for such a restriction is their presence as residues in milk over a long period of time. From the above it is obvious that control of residues of veterinary drugs in milk is a must and that it is done in Slovenia under the veterinary sanitation control of food of animal origin.

Material and methods

Samples

Milk samples were taken at collection sites at various dairy plants in Slovenia. The collection programme was performed according to the plan drawn up by the Veterinary Administration of the Republic of Slovenia, following our

official guidelines (Ur. list SFRJ, 1989). We prepared sub-samples from milk samples brought to the laboratory. Analyses of antibiotics were performed immediately or the following day, and sub-samples were stored at 4°C. For other analyses the sub-samples were kept under -20°C until the day of analysis. Samples were thawed and well homogenised before analysis.

Antibiotics

Antibiotics in milk samples were detected using Delvotest SP and confirmed by microbiological assays. Delvotest SP was used for the detection of penicillins; microbiological assays were used for the detection of tetracycline, oxitetracycline, chlortetracycline, streptomycin, erythromycin, neomycin and other antibiotics. Delvotest SP was commercially available from Gist-Brocades. Samples of milk were incubated with *Bacillus stearotermophilus var. calidolactis* (in sporulated form) at 64°C for 2.5 hours. As the bacteria grew, they released lactic acid that reduced the pH and changed the colour of an indicator built into the kit from purple to yellow. If the milk contained anti-microbial substances, these substances inhibited the growth of the bacteria and the indicator remained purple or, depending on the concentration of inhibitory substances, became partly yellow and partly purple. This test detected most anti-microbial residues but with varying levels of sensitivity. For microbiological assays (SS, 1974) the antibiotic seed agar (Biolife®) was used, with an appointed test culture added that was sensitive to the antibiotic being assessed. The test cultures were *Sarcina luteae* ATCC 9341 for the determination of penicillins, *B. subtilis* ATTC 6633 and S. luteae ATCC 9341 for the determination of streptomycin, *S. luteae* ATCC 9341 and *S. luteae* ATCC 15957 for the determination of erythromycin, *B. cereus* ATTC 11778 and *St. epidermidis* ATCC 12228 for the detection of tetracyclines, *S. luteae* ATCC 9341 for the detection of tylosine, *B. subtilis* Merck for the detection of gentamicine, and *St. epidermidis* ATCC 12228 and *S. luteae* ATCC 9341 for the determination of neomycin. The milk samples were warmed at 80°C for 5 minutes and inoculated into metal cylinders placed on the prepared seeded agar. The inoculum penetrated into the agar and, if antibiotics were present in the sample, the growth of the test bacteria was inhibited. The detection limits were different for each antibiotic and were as follows: penicillin 0.013 I.E., streptomycin 0.1 μg/mL, erythromycin 0.05 μg/mL, tetracycline and oxitetracycline 0.05 μg/mL, chlortetracycline 0.01 μg/mL, tylosine 0.1 μg/mL, neomycin 0.25 μg/mL and gentamycine 0.05 μg/mL.

Sulfonamides

Before the extraction of sulfonamides from milk samples using chloroform-acetone (Smedley and Weber, 1990), the internal standard (sulfapyridine) was added. Evaporation of the organic phase followed. Residues were

dissolved in an aqueous potassium phosphate solution and fatty residues were extracted into hexane. The aqueous layer was collected for HPTLC. Pre-adsorbent layer silica gel plates were used for chromatography. Developed plates were treated with fluorescamine and scanned using a fluorescence densitometer (Van Poucke et al., 1991). With the above analytical procedure we were able to monitor: sulfanilamide, sulfachloropyridazine, sulfachloropyrazine, sulfadimethoxine, sulfadoxine, sulfadiazine, sulfisoxazole, sulfaguanidine, sulfamethoxydiazine, sulfamonomethoxine, sulfamoxole, sulfamethoxypyridazine, sulfamethoxazole, sulfamethazine, sulfapyridine, sulfaphenazole, sulfaquinoxaline, sulfamerazine and sulfathiazole. Limits of detection ranged from 10 to 20 ng/mL depending on the individual sulfonamide.

Chloramphenicol

After the addition of an internal standard (meta chloramphenicol) to milk samples, fatty residues were extracted into hexane. Samples were cleaned-up with extraction on Extrelut® columns (Keukens et al., 1986) and chloramphenicol was eluted in ethyl acetate. The eluate was evaporated to dryness and redissolved in water. Fat was separated with hexane again. Chloramphenicol residues were derivatised by silylation to form a thermally stable product (FSIS, 1991), which was analysed by capillary GC with EC detector. The analytical column was HP-5 (35 m x 0.2 mm x 0.33 μm), with cross-linked 5% phenyl methyl siloxane as the stationary phase. Nitrogen was used as a carrier and make-up gas. The temperatures of the GC instrument were as follows: injector 230°C; detector 350°C; column 80-278°C. The limit of detection was 2 ng/g.

Ivermectin

Ivermectin is a mixture of two homologues containing not less than 80% 22,23-dihydroavermectin B_{1a} (H_2B_{1a}) and not more than 20% 22,23-dihydroavermectin B_{1b} (H_2B_{1b}). H_2B_{1a} is used as a marker substance for residue analysis because of the greater proportion in the parent drug and often slower excretion from organisms. Milk samples were extracted using acetonitrile and cleaned-up with SPE on C8 columns (Nordlander and Johnsson, 1990; Beek, 1992). Eluate in acetonitrile was evaporated to dryness under nitrogen. Ivermectin residues were derivatised with trifluoroacetic anhydride and N-methylimidazole to form a highly fluorescent derivative (De Montigny et al., 1990; Beek, 1992), which was analysed using isocratic HPLC on the reversed and deactivated stationary phase with fluorescent detection. The limit of detection was 0.15 ng/g.

Results and discussion

The results of the four-year control (1995-1998) of residues of veterinary drugs in Slovenian milk are presented in Tables 1 to 4.

Residues of antibiotics have been controlled in our laboratory since 1977. As we can see from Table 1 only 19 of the 3358 samples of milk were positive (i.e. antibiotics were present). Over the four-year period 99.4% of analysed milk samples were found to contain no antibiotics.

Table 1: Results of examinations and number of examined Slovenian milk samples on antibiotics in the period from 1995 to 1998
Tablica 1: Rezultati ispitivanja uzoraka mlijeka u Sloveniji na prisutnost antibiotika u periodu od 1995. do 1998.

Year Godina	No. of samples Br. ispitivanih uzoraka	No. of negative samples Br. negativnih uzoraka	No. of positive samples Br. pozitivnih uzoraka
1995	405	399	6
1996	610	601	9
1997	1499	1497	2
1998	844	842	2
Total Ukupno	3358	3339	19

Antibiotic residues can appear in milk via several routes: mastitis treatments, injectables, feed contaminated with antibiotics, inappropriate feeding of antibiotics, bolluses used during post-calving to prevent infections, and the addition of drugs to drinking water. We can be satisfied with the results as we know that more than 70 different veterinary medicinal products containing one or more antibiotics are on the market in Slovenia. The withdrawal periods for milk range from one to 17 days or even more, depending on the type of antibiotic and administration.

Despite the large number of veterinary medicinal products containing antibiotics that can be used for treating milk-producing animals and the various withdrawal periods, we can conclude that the use of antibiotics is controlled and that, generally speaking, the withdrawal periods are respected.

Until recently in Slovenia (Ur. list RS, 2000) the law did not prescribe MRL values for individual antibiotics but stipulated that no antibiotics may be present in the sample according to the prescribed or recognised analytical methods (Ur. list SFRJ, 1983). A so-called zero tolerance policy was in force so further identification of antibiotics was not carried out.

Sulfonamides are also widely used in veterinary medicine for prophylactic purposes and for the treatment of various infections in food-producing animals. There are various veterinary medicinal products on the Slovenian market containing one or two of 11 different sulfonamides. For milk-producing animals there are veterinary medicinal products on the market that may contain one of 8 possible sulfonamides. For this reason a method capable of determining all possible sulfonamides in milk samples was necessary. Despite this the

permissible level of sulfonamide residues in milk is 100 ng/mL (Ur. list SFRJ, 1987), which is the same as the MRL value in the European Union (Commission Regulation EC No 281/96). We can see from Table 2 that no residues were found in any of the 406 analysed milk samples between 1995 and 1998. Control of residues of chloramphenicol in Slovenian milk was introduced in 1996.

Table 2: Sulfonamides in Slovenian milk in the period 1995 to 1998, and distribution into classes with regard to sulfonamide content [ng/mL]
Tablica 2: Prisutnost sulfonamida u uzorcima mlijeka u Sloveniji u periodu od 1995. do 1998. te njihova podjela u razrede s obzirom na sadržaj sulfonamida [ng/mL]

Year Godina	No. of samples Br. uzoraka	No. of negative samples Br. negativnih uzoraka <10 ng/mL	10 < 100 ng/mL	100 < 200 ng/mL
1995	51	51		
1996	137	137		
1997	107	107		
1998	111	111		
Total Ukupno	406	406		

Chloramphenicol is a broad-spectrum antibiotic with very effective antibacterial properties. In susceptible individuals it can cause serious side-effects; these include aplastic anaemia and bone marrow aplasia, which are both dose independent. For these toxicological reasons its use is allowed in human medicine only in exceptional circumstances; however, it is banned for food-producing animals because of the possible occurrence of residues. Despite the fact that the ban on the use of chloramphenicol for food-producing animals was issued recently in Slovenia (Ur. list RS, 1999), there have been no registered veterinary medicinal products for food-producing animals containing chloramphenicol since 1989. In the European Union such a ban was brought into force with Commission Regulation (EU) No 2701/94 in 1994. Chloramphenicol is classified among the substances in Annex IV of the Council Regulation (EEC) No 2377/90 for which, on the grounds of consumer safety, no MRLs may be attributed. Because of possible illegal use, effective control of its residues in food of animal origin, including milk, is essential with very low limits of detection. In 1996, 1997 and 1998 we analysed 217 milk samples for chloramphenicol; in 1997 one milk sample contained 4.6 ng/g chloramphenicol (Table 3). The presence of chloramphenicol in the milk sample indicated that a veterinary medicinal product containing chloramphenicol was illegally used for milk-producing animal(s) for human consumption and that control of chloramphenicol residues must be carried out to protect consumers.

Table 3: Chloramphenicol in Slovenian milk in the period from 1995 to 1998, and distribution into classes with regard to chloramphenicol content [ng/g]
Tablica 3: Prisutnost kloramfenikola u uzorcima mlijeka u Sloveniji u periodu od 1995. do 1998. te njihova podjela u razrede s obzirom na sadržaj kloramfenikola [ng/g]

Year Godina	No. of samples Br. uzoraka	No. of negative samples Br. negativnih uzoraka <2 ng/g	2< 5 ng/g	5< 20 ng/g	> 20 ng/g
1995					
1996	129	129			
1997	68	67	1[a]		
1998	20	20			
Total	217	216	1		

[a] Chloramphenicol content in the sample was 4.6 ng/g
[a] Sadržaj kloramfenikola u uzorku iznosio je 4,6 ng/g

In 1998 the control of ivermectin residues in milk was introduced in Slovenia. Ivermectin is a novel broad-spectrum antiparasitic drug which is highly effective against both internal and external parasites, specifically nematodes and arthropods. It is derived from fermentation of the micro-organism *Streptomyces avermitilis*, followed by hydrogenation. In Slovenia we have 5 registered veterinary medicinal products with ivermectin for therapeutic and prophylactic use in cattle, sheep, pigs and horses. Its use is prohibited during the lactation period of food-producing animals, so any residues in milk would be a violation. From this reason, analytical methods for the determination of ivermectin residues in milk should have a low detection limit (the detection limit of our method was 0.15 ng/g). As can be seen from Table 4 we analysed 50 milk samples and none of them contained any ivermectin residues.

Table 4: Ivermectin in Slovenian milk in the period from 1995 to 1998, and distribution into classes with regard to ivermectin content [ng/g]
Tablica 4: Prisutnost ivermektina u uzorcima mlijeka u Sloveniji u periodu od 1995. do 1998. te njihova podjela u razrede s obzirom na sadržaj ivermektina [ng/g]

Year Godina	No. of samples Br. uzoraka	No. of negative samples Br. negativnih uzoraka <0.15 ng/g	0.15 < 20 ng/g	> 20 ng/g
1995				
1996				
1997				
1998	50	50		
Total Ukupno	50	50		

Conclusions

From the results of the analysis of samples of Slovenian milk for residues of veterinary medicinal products between 1995 and 1998, we can conclude that residues of veterinary drugs are not involved. Antibiotics were found in 19 samples, which means that 0.6% of 3358 analysed milk samples were positive. If we take into account the fact that antibiotics are among those veterinary medicinal products widely used for treating mastitis infections as well, the low percentage of positive samples is understandable. If we also take into account the fact that no MRL values for antibiotics were in force in Slovenia and that these 19 positive samples can have any amounts of antibiotics (even lower than the MRL values), we can conclude that the results are no cause for concern. Residues of sulfonamides in milk have not presented any problem in Slovenia since all 406 examined samples were negative (< 10 or <20 ng/mL). Control of the presence in milk of prohibited substances for milk-producing animals began in 1996. The residue monitoring of chloramphenicol was introduced and a total of 217 samples were analysed. Only in one sample (0.5%) did we find chloramphenicol (4.6 μg/kg), which represented an instance of the illegal use of the substance for treating food-producing animals. In 1998, 50 samples of milk were also examined for ivermectin and no residues were found.

OSTACI VETERINARSKIH LIJEKOVA U UZORCIMA SLOVENSKOG MLIJEKA ANALIZIRANIH U RAZDOBLJU OD 1995. DO 1998.

Sažetak

U Sloveniji je provedeno ispitivanje prisutnosti ostataka veterinarskih lijekova u uzorcima mlijeka. Praćenje prisutnosti antibiotika započeto je 1977. godine. U razdoblju od 1995. do 1998. ispitano je 3358 uzoraka mlijeka od kojih je većina (99,4%) bila negativna na prisutnost antibiotika. Prisutnost antibiotika određena je samo kod 19 uzoraka. Određivanje sulfonamida u mlijeku započeto je 1995. godine. Tijekom četiri godine ispitano je 406 uzoraka od kojih su svi bili negativni na prisutnost sulfonamida. Ispitivanja su nastavljena ponovno 1996. godine, određivanjem kloramfenikola. U ovom slučaju ispitano je 217 uzoraka, od kojih je jedan uzorak sadržavao 4,6 μg/kg kloramfenikola. Prisutnost ovog antibiotika nije dozvoljena u proizvodima animalnog podrijetla. Godine 1998. ispitano je 50 uzoraka mlijeka na prisutnost ivermektina. Svi uzorci su pokazali negativnu reakciju na prisutnost ovog antibiotika.

Ključne riječi: uzorci mlijeka, analiza, prisustvo antibiotika.

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