JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 12199–12203 12199
DOI:10.1021/jf102351p

Depletion of Long-Acting Ampicillin in Goat Milk following Intramuscular Administration

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Although goat milk production represents today a very small percentage of the world milk market, this percentage has been growing continuously during the past 20 years. Goat milk is the basic milk supply in many developing countries and provides tasteful derivative products in developed countries. Goats, as well as all milk-producing animals, can be affected by mastitis, but goats being considered a minor species, few drugs are specifically registered for these animals; most, at least for mastitis treatment, are usually tested and registered for use in cows. This situation leads often to the adoption for goat milk of withdrawal periods defined for cows even if these extrapolations prove almost never valid for goats. In the present study, the elimination of the β-lactam antibacterial agent ampicillin in goat milk was investigated. Ampicillin was chosen because it is one of the most common antibiotics used by goat farmers against mastitis due to the fact that it is well tolerated and has short elimination times in cows. Goats were treated with long-acting ampicillin at 15 mg (kg of body weight)$^{-1}$ by double intramuscular injection at 72 h interval. Milk was collected in a 12 h milking scheme. The method used to determine the levels of ampicillin in goat milk was based on a liquid−liquid extraction of this drug from the matrix, successive derivatization with formaldehyde, and final separation by HPLC with fluorescence detection. The results point out a slow depletion of ampicillin and, consequently, a withdrawal period (13 milkings) longer than that extrapolated and authorized for cows and sheep.

KEYWORDS: Ampicillin; goat milk; HPLC-FLD; depletion; withdrawal time

INTRODUCTION

Goat milk has a two-fold and paradoxical role in human nutrition. On the one hand, it replaces cow milk as feeding source for malnourished people in many countries of the third world; on the other hand, its derivative products, in particular yogurt and cheeses, fill the demand for specialty food of some consumers of developed countries. Moreover, goat milk, thanks to its nutritional characteristics, is prescribed for people suffering from cow milk intolerance/allergies and gastrointestinal disorders even if this nutritional function is still inconsistent (*1*−*4*). This growing importance is also reflected in the largest animal number increase for goats during the past twenty years (+60%) and the largest increase in goat milk production tonnage (+ 51%) compared to other milk-producing farm animals (*5*). Obviously, sustainable growth needs good management and veterinary practices capable of controlling the recurrent problems arising from bacterial infectious diseases.

Mastitis is known as the most common disease in goat breeding (*6*). Treating mastitis-affected animals with antibiotics is a veterinary practice to cure the disease and prevent its diffusion through the herd (*7*). Independently from the administration route, the administered antibiotic (or its metabolite) may be excreted into milk for a period of time depending on different factors, basically drug, dosage, administration route, vehicle, animal species, and animal health status. Antibiotic residues in milk are of great concern to dairy farmers, milk processors, consumers, and regulatory agencies. Therefore, the Commission of the European Union has established and periodically reviews drug maximum residue levels (MRLs) in foods. An MRL is defined as the maximum concentration of residue resulting from the use of a veterinary medicinal product that may be accepted by the European Union to be legally permitted or recognized as acceptable in or on a food (*8*).

β-Lactams are effective antibacterial drugs widely used both in human and in veterinary medicine against various systemic bacterial infections (*9*, *10*). Ampicillin, a molecule belonging to the β-lactam antimicrobial family, has received growing attention because of its efficacy and tolerability for the treatment of mastitis in goat breeding (*11*). The Commission of the European Union has established an MRL of 4 μg kg^{-1} for ampicillin in milk of all milk-producing species (*12*).

The use of antibiotics and chemotherapeutic agents in animals reared for human consumption should be based on toxicological and pharmacokinetic data obtained in the specific animal species considered. On the contrary, due to the costs of the experimentation,

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Published on Web 11/12/2010

most of the antibiotics are usually tested on the most important animal species in terms of production. For this, most of the data on drugs intended for milk-producing animals are obtained from studies performed on cows. In particular, for β-lactams, some microbiological, metabolic, and pharmacokinetic aspects have been reported in previous studies with animals, but very few data on pharmacokinetic in goats reared under field conditions are available (*13*−*18*). Depletion time is an important parameter that states the time needed before the antibiotic disappears from the milk or decreases under a fixed value and objectively defines when the milk of treated animals can be considered safe for consumption. Drug depletion time is species dependent and so should be taken into account also for minor species of ruminant mammals.

The aim of this study was thus to determine residue persistence in goat milk following ampicillin therapy and to calculate the suitable withdrawal time.

The analytical determination of ampicillin levels was carried out by a high-performance liquid chromatography method with fluorescence detection (HPLC-FLD), developed on the basis of a previously published method (*19*) with some modifications. The modified method has been in-house validated for the determination of ampicillin residues in goat milk, following the requirements of European Commission (EC) Decision No. 657/2002 (*20*).

MATERIALS AND METHODS

Reagents and Chemicals. Ampicillin standard, purity = 98.1%, was purchased from Sigma (Milan, Italy). Standard stock solutions were prepared by dissolving 10 mg of standard in 0.05 M sodium acetate buffer solution (pH 5) and adjusting the final volume to 10 mL. These solutions were stable at −20 °C for 6 months. Working standard solutions were prepared by scalar dilutions 1:10 (v/v) of the stock solution with 0.05 M sodium acetate buffer solution (pH 5). Working solutions were stable at 4 °C for 2 weeks.

Albipen L.A. (a commercial long-acting drug of which the active ingredient is ampicillin) was purchased from Intervet International B.V. Boxmeer, The Netherlands. Acetonitrile and water were of HPLC grade, and the other reagents used were of analytical grade.

Animals. Twenty-four healthy Saanen goats, all from the third to the fourth month of lactation, with an average weight of 43.5 ± 2 kg, were used in the investigation. On their arrival at the farm, the animals were examined, quarantined, and reared in accordance with standard Good Farming Practices (GFPs).

Before the drug administration was begun, the animals were acclimatized for a period of 30 days. During this time the goats did not undergo any pharmacological treatment. Subsequently, the goats were randomly divided into two groups (A and B) of 4 and 20 animals, respectively. Group A was employed as control.

Ampicillin was administered as Albipen L.A. to the group B animals, whereas control group (A) animals were treated with the administration medium (physiologic solution) during the same period. Because no information on ampicillin therapeutic regimens for goats was available, the dose and route of administration were chosen following the drug insert descriptions and suggestions for therapeutic treatments for sheep. For this species the treatment provides intramuscular injection of 15 mg (kg of body weight)$^{-1}$, which is repeated after 72 h. Thus, the same dosage was chosen for goat, and the drug was administered by two deep intramuscular injections (72 h interval) in the neck region, using a syringe with a sterile hypodermic needle. Each goat was weighed to calculate the exact dose to administer to each individual.

Sample Collection. The goats were hand-milked twice daily at 12 h intervals (8 a.m. and 8 p.m.) during a period of 10 days (from hour 0 to hour 240). The zero time coincided with a milking made shortly before the first injection of ampicillin. Individual milk related to each goat and milking was singularly analyzed. The samples (100 mL/milking session/group) were collected in disposable plastic containers and kept at 5 °C until the day after when they were analyzed.

Sample Preparation and Analysis. The compositional analyses of all milk samples collected were carried out by MilkoScan Minor 4 (Foss, Padua, Italy).

The methodology used to determine the levels of ampicillin in goat milk was based on the extraction of this drug from the matrix, successive derivatization, and final separation by HPLC-FLD. The sample pretreatment procedure and the chromatographic conditions were similar to those described by Ang and Luo (*19*), except for some modifications both to the extraction procedure and to the chromatographic conditions.

Briefly, 0.1 mL of water, 0.5 mL of trichloroacetic acid (TCA), and 0.5 mL of acetonitrile were successively added to 2.5 mL of the homogenized milk sample. After agitation on a vortex mixer for 2 min and sonication for 10 min at room temperature, the sample was centrifuged at 5000*g* for 10 min. The supernatant was then filtered on Whatman grade no. 42 quantitative filter paper, and 2 mL of filtrate was transferred into a 15 mL flask. The sample solution was reacted with 0.6 mL of TCA, 30% (w/v), and 1 mL of formaldehyde, 7% (w/v) at 100 °C for 30 min to form fluorescent derivatives. The mobile phase was added to the sample solution until a known volume (3 or 4 mL) and, after filtration through 0.45 μm pore size nylon filters, 50 μL of the final solution was injected into an HPLC-FLD system, assembled as follows: a pump with an autosampler device (model Alliance 2690; Waters S.p.A., Vimodrone, Milan, Italy) fitted with a KR100-5C18 column (250 × 4.6 mm, 5 μm) packed by Kromasil (Bohus, Sweden); a Croco-cil column oven heated to 40 °C; and a fluorescence detector (model 474; Waters) set at 354 and 445 nm as the excitation and emission wavelengths, respectively.

The mobile phase consisted of 15% phosphate buffer (0.01 M, pH 5.6) and 85% acetonitrile with a flow rate of 1.5 mL/min in isocratic mode.

Validation of In-House Method. Before sample analysis, the HPLC method described above was validated in-house according to the recommendations of Commission Decision (EEC) No. 657/2002 (*20*). To achieve the in-house validation of the analytical method, the following parameters were considered: recovery, repeatability, specificity, linearity, limit of detection (LOD), and limit of quantification (LOQ).

In the absence of any certified reference material (CRM) for ampicillin in milk, the method accuracy was evaluated on the basis of recoveries obtained from in-house standard materials (fortified samples). Briefly, a blank milk sample of farmed goat (previously analyzed and found not to be contaminated) was fortified with the ampicillin at five different levels (0, 5, 10, 20, and 50 μg L^{-1}).

Precision, expressed as repeatability, was calculated by repeated analyses on the same sample sets as used for recovery tests, with the only difference that independent samples were re-extracted and analyzed on two other occasions to calculate interday repeatability. Linearity was determined by analyzing HPLC−FLD standard curves (standard solutions) and matrix calibration curves (the above-mentioned fortified samples).

Even if ampicillin in goat milk has an MRL (4 μg kg^{-1}), the LOD and LOQ for ampicillin in goat milk were calculated instead of CCα and CCβ for better achieving the aim of this study. Twenty blank goat milk samples were analyzed, and the signal-to-noise ratios relative to the ampicillin chromatographic time windows were calculated. Three times the signal-to-noise ratio was used as decision limit. LOQ was estimated by analyzing 20 blank goat milk samples fortified with ampicillin at the LOD limit: LOD limit values, plus 1.64 times the standard deviation of within-laboratory reproducibility relative to the measured contents, equals the detection capability (β = 5%)

Finally, to comply with internal quality control (IQC) procedures, two control samples (house reference materials) were inserted into each analytical batch made up of six samples. The individual values obtained for control samples were plotted on a Shewhart control chart during the entire duration of the study.

Statistical Analysis. For the establishment of withdrawal time for goat milk, the harmonized method suggested by the European Agency for the Evaluation of Medicinal Products (EMEA) in its guidance for the determination of withdrawal periods for milk has been applied (*21*). The approach is named the Time To Safe Concentration (TTSC) method and calculates a tolerance limit on the milking number per animal. This last parameter is the time necessary for the residue level in the milk of most animals to arrive at the safe concentration (MRL). The method adopts a log-normal distribution and adjusts the possible increasing concentrations found during the depletion period by means of a monotonic regression. A second monotonic regression smoothes the correlation between the resulting withdrawal time and MRL with a confidence level of 95%.

Table 1. Performance of the Analytical Method for the Determination of Ampicillin in Milk

fortification level (μg kg^{-1})	recovery[a] (%)	intraday repeatability[b] CV (%)	interday repeatability[c] CV (%)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
5	110	5.1	6.2	1.5	2.2
10	92	6.1	6.4		
20	86	1.8	3.3		
50	80	6.1	6.5		

[a] Values refer to 9 independent samples. [b] Values refer to 9 independent samples analyzed on a single day. [c] Values refer to 18 independent samples analyzed on three different days (9 samples analyzed per day).

RESULTS AND DISCUSSION

Method Validation Parameters. The developed analytical method proved to be very adequate within the scope of the present investigation. The performance values (**Table 1**) satisfy the criteria fixed by the 2002/657/EC Decision (*20*).

Recovery data for ampicillin in goat milk were acceptable, with values ranging from 80 to 110% depending on the fortification level.

The coefficients of variation (CV) values for intraday and interday repeatability ranged from 1.8 to 6.1% and from 3.3 to 6.5%, respectively. These data indicate that both intraday and interday repeatabilities are good, because all CV values are below the recommended limits based on the Thompson equation for fortification levels ($\leq$100 μg kg^{-1}).

LOD and LOQ values were equal to 1.5 and 2.2 μg kg^{-1}, respectively.

The calculated matrix calibration curves showed a fair linearity over the whole range of tested concentrations (0−50 μg kg^{-1}) with a coefficient of determination (R^2) equal to 0.9978.

Depletion from Milk. The composition of milk samples (n = 20 animals) was rather homogeneous with average percent values of 3.56 ± 0.07 for proteins, 4.52 ± 0.20 for lactose, 4.34 ± 0.40 for fat, 13.0 ± 0.4 for total solids, and 0.79 ± 0.04 for ash.

Results of ampicillin depletion at different times in goat milk are shown in **Table 2**. Some interesting considerations derive from our results. With regard to ampicillin concentration in goat milk two major peaks have been observed during the time. The first one (17 μg kg^{-1}) occurs 36 h after the beginning of the drug treatment (first intramuscular injection). The second peak, corresponding to the highest ampicillin average concentration (45 μg kg^{-1}), arises at 84 h, which is 12 h after the end of drug treatment (second intramuscular injection). This concentration peak is obviously greater because the second ampicillin administration is made when ampicillin residues of the first administration are still present in goat milk.

The depletion trend of ampicillin in goat milk after 84 h is particularly noteworthy. As can be seen from **Table 2**, the ampicillin concentration does not smoothly decrease with time but presents three minor peaks at 120, 144, and 168 h. Because all three peaks were correlated with evening milking, this apparent discrepancy can be likely explained by daily variation of the nutritional composition of goat milk. In fact, the latter is found to have significant daily variation associated with main nutrients. Previous studies have verified these fluctuations in goat milk for fat, proteins, and total solids with a greater percent content of all three nutrients in morning milk (*22*, *23*). This daily variability becomes especially marked after the third month of lactation when maximum nutrient concentrations are reached. Consequently, the concentrations of unbound and protein-bound ampicillin vary according to the difference of composition between morning and evening goat milk (*24*). This depletion trend gives rise to the fact that the milk from subsequent milkings may be alternately unsafe and safe or better above or below the MRL (**Table 2**).

Table 2. Ampicillin Depletion at Different Times in Goat Milk after Two Administrations of the Long-Acting Drug (15 mg (kg of Body Weight^{-1})) in a 72 h Interval

time (h)	ampicillin average concentration in goat milk[a] (μg kg^{-1})	CV (%)
0[b]	<LOQ	
12	2.2 ± 1.0	45
24	5.5 ± 2.1	38
36	17.1 ± 5.0	29
48	9.3 ± 2.8	30
60	9.5 ± 2.4	25
72[c]	27.5 ± 7.3	27
84	45.4 ± 7.1	16
96	21.1 ± 3.2	15
108	9.4 ± 2.5	27
120	7.4 ± 2.5	34
132	2.9 ± 0.7	24
144	7.1 ± 2.3	32
156	2.5 ± 0.5	20
168	6.0 ± 1.3	22
180	<LOQ	
192	<LOQ	
204	<LOQ	
216	<LOQ	
228	<LOQ	
240	<LOQ	

[a] Values are reported as concentration means ± standard deviation (n = 20 animals). [b] First ampicillin injection. [c] Second ampicillin injection.

Withdrawal Period. As recommended by the EMEA, the first milking utilizable for withdrawal time calculation is that related to milk collected 12 h after the last drug administration. In this study the first milking taken into consideration corresponds, thus, at the 84 h milking, 12 h after the second intramuscular injection.

The withdrawal period has been calculated using the statistical program recommended by the EMEA and downloadable from the same EMEA Website. Using this software, a withdrawal period of 13 milkings has been interpolated for milk of goat treated with a double injection of ampicillin (72 h interval) at 15 mg (kg of body weight)$^{-1}$. The estimated withdrawal period was significantly longer than that recommended by the pharmaceutical company for cow and sheep, the latter being equal to six milkings for falling below the MRL. Also, by applying a linear regression method (SCLR), the corresponding withdrawal period would be anyway longer than six milkings, giving as a result eight milkings.

This outcome is remarkable because it proves once again that the pharmacokinetics of a single drug changes, also to a great extent, in similar animals, that is, the case of sheep and goat. Consequently, the withdrawal period of a certain drug estimated for a given species cannot simply be extrapolated to another species. The withdrawal period calculated in this study was also longer than the precautionary withdrawal period of 12 milkings established for Albipen L.A. (off-label use) by the annex II of the Italian Ministerial Decree of March 4, 2005, which acknowledges the European Directive 2001/82/EC (*25*, *26*).

Previous studies have been carried out on the excretion rate of antibiotic in milk, but different drugs, species, administration routes, doses, and specimens as well as different methods for withdrawal period calculation were applied. For this reason, it is difficult to compare our results with other data available in the scientific literature. However, our study confirms data of previous studies that pointed out very different drug pharmacokinetics

among cow, sheep, and goat. In particular, almost all of the studies carried out on several drugs reported withdrawal periods for goat milk longer than those verified for cow and sheep milk (*17*, *18*). Karzis et al. (*17*) found a similar lengthening of withdrawal times for an ampicillin treatment of goats if compared with the withdrawal periods recommended for use in cows. Analytical method and administration route were different, but results showed a mean withdrawal period of eight milkings that was significantly longer than that estimated for cows (five milkings).

The availability of additional safe information for minor species such as the goat is important for consumer health. When correct withdrawal periods become known, improper uses of drugs are generally reduced (*27*, *28*) and incidences of antibiotic resistance are less frequent (*29*). In this specific instance, if therapy with β-lactams is made on goats following the legal indications assessed for other animal species, the longer excretion period would lead unintentionally to ampicillin residues in milk at concentrations above its MRL with a jeopardizing effect of human health. In fact, β-lactams residues can foster drug-resistance phenomena (*30*, *31*) and, if absorbed at certain concentrations, might cause adverse effects in humans, above all, children and teenagers (*32*−*34*). This must be taken into consideration because goat milk is normally targeted to vulnerable segments of the population and suggested by pediatricians in cases of allergies or intolerances.

It must also be considered that a large proportion of the whole goat milk production is for cheese production. This implies two other aspects: the technological defects in the starter activity, with a consequent loss in the quality of the product, and/or the conjecturable further concentration of the residue contaminant in the cheese, with a magnified risk for the consumer. In particular, ampicillin residues in goat milk can affect yogurt and whey-derived products, for example, ricotta and brunost. In fact, being a hydrophilic compound, the ampicillin concentrates in whey.

All of these aspects underscore the importance of the appropriate evaluation of the depletion time of the administered antibiotics.

ABBREVIATIONS USED

HPLC-FLD, high-performance liquid chromatography with fluorescence detector; MRL, maximum residue level; LOD, limit of detection; LOQ, limit of quantification; GFP, good farming practice; CCα, decision limit; CCβ, detection limit; TCA, trichloroacetic acid; EMEA, European Medicines Agency; TTSC, time to safe concentration; SCLR, safe concentration linear regression; IQC, internal quality control.

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Received for review June 18, 2010. Revised manuscript received October 20, 2010. Accepted October 31, 2010.