Amoxicillin Resides in Milk of Holstein Cows With Double or Triple Intramammary Administration of Amoxicillin-Potassium Clavulanate

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ABSTRACT

The aim of this study was to determine differences in residue elimination of amoxicillin (AMX) in cow´s milk, after intramammary administration of a pharmaceutical preparation of AMX plus potassium clavulanate (CLV), either in healthy or in spontaneous cases of clinical mastitis, and following label instructions or administering the preparation in an extra-label manner. Twelve lactating healthy Holstein/Friesan cows in production and negative to clinical mastitis were treated with a commercial brand of intramammary AMX/CLV (300 mg of AMX and 60 mg of CLV), administered every 12 h for 3 consecutive days (CL group), as recommended on the label. Twelve cows with clinical mastitis, determined by California test, somatic cell count, microbiological culture and physical aspect of the milk, were dosed as group CL (Exp-L group). Twelve cows, also affected with mastitis, were treated with double dose for three days (Exp-EL1 group). Finally, a fourth group of 12 cows with mastitis were treated in an extra-label manner by tripling the referred dose (Exp-EL2 group). After the last administration, milk samples from all the

treated/affected glands were obtained every 12 h for 7 days. Amoxicillin concentrations in milk were determined by HPLC. Results showed that in CL and Exp-L groups, mean number of milkings required to quantify AMX below the official maximum residue level (MRL) in Mexico (4 μg/L), was 7 (84 h). In contrast, when double or triple dose of the preparation was administered in groups Exp-EL1 and Exp-EL2 groups, a mean of 11 milking times (132 h) were required to set AMX concentrations below the referred MRL, suggesting a zero order elimination kinetics. There is a statistically significant difference between the former and the latter groups. The amoxicillin elimination half-lives were 46.97 h and 66.92 h for CL and Exp-L groups and 71.46 h and 85.43 h for Exp-EL1 and Exp-EL2, respectively. These findings stress the need to comply with label instructions.

INTRODUCTION

Residues of antibacterial drugs in cow´s milk are regarded as a public health threat (Salter, 2003; Donoghue, 2003; Karis et al., 2007). Additionally, residues of antibacterial drugs modify processing of dairy products (Sawant et a., 2005; Karis et al, 2007, Germán, 2012). Based on these premises, private companies and regulatory agencies worldwide have set residue detection monitoring programs. The use of on-site detection kits (Charm , SNAP and Delvotest®) for antibacterial drug detection, has greatly improved milk quality in many dairies. Yet, according to some authors, unlawful residue levels of antibacterial drugs in milk, is a constant finding, rather than an exception (Gutiérrez et al., 2005; Bogialli et al, 2007; Kantiani et al, 2009). Accountability of such residues for health problems in humans is not so clear cut, yet it is believed to range from an increase in bacterial resistance of pathogens in humans, to direct health problems in consumers such as allergic reactions (van de Bogaard et al, 2000; Cabello, 2004; Cabello, 2006). For example, residues of amoxicillin/clavulanic acid in animal products have been associated in general with an increase in bacterial resistance, higher risk of gastrointestinal adverse reactions, hepatotoxicity, diarrhea, and intractable urinary tract infections by Escherichia coli and Salmonella spp. (Hensel et al, 2005, Salvo et al, 2007; Kemper, 2008).

In Mexico, maximum residue levels (MRLs) of antibacterial drugs in milk are usually in agreement with international criteria (FDA - USA, Codex Alimentarius, EMEA), and as it happens in many countries, establishment of clearance patterns and withholding time by a sponsor of a given antibacterial-drug preparation is carried out in healthy individuals from the target species. In this particular case, residue elimination in milk after treatment with an antibacterial preparation, intended for intramammary administration, is carried out in healthy cows. Trials in cows suffering mastitis are almost never done. Furthermore, antibiotic clearance rate after overdosing cows, are rare at best. For amoxicillin (AMX) and potassium clavulanate (CLV) in Mexico, official MRL values are 4 μg/kg and 200 μg/kg, respectively (EMEA, 2002). For a commercial brand of AMX/CLV in Mexico, a withdrawal period of 3 days (6 milkings, one every 12 hours) has been accepted. However, extra-label overdosing of such preparation in a given mammary gland, based on the clinical perception that more of these antibacterial drugs are necessary, may modify the clearance pattern of AMX/CLV preparation. Considering the above, the present study was set to test the hypothesis that Holstein/Friesian cows show a different pattern of elimination of AMX in milk when overdosed (twice or thrice), in comparison to healthy cows or cows suffering from mastitis that received label recommended use of this drug.

MATERIAL AND METHODS

Charm®; Rapid test based on the ligand assays using biological receptor.
SNAP®; Rapid test based on the ligand assays using antibodies configured in an enzyme-linked immunoassay.
Delvotest® Rapid test based on the inhibition of growth of microbial test organisms.

All study procedures and animal care activities were conducted in accordance with the Institutional Committee of Research, Care and Use of Experimental Animals of the National Autonomous University of Mexico (UNAM), according to the Mexican Official Regulation NOM-062-ZOO-1999 (1999). This trial was carried out at the milk dairy "El Puente" located in the State of Mexico, having 500 Holstein/Friesian cows in production.

Twelve healthy cows were used to set a control group (CL group). Their status was based on an electronic somatic cell counter < 200,000 cells/ml; negative to California mastitis test (CMT) and to microbiological culture and lack of signs of the disease, plus a thorough physical examination of the udder. In the group of cows with udder infection (groups Exp-L, Exp-EL1, Exp-EL2), the following inclusion criteria was set for 36 Holstein/Friesian cows (12 animals per group) diagnosed as having a spontaneous case of mastitis: sign of inflammation in at least one quarter of the mammary gland, modification of physical aspect of milk when passing through a dark cloth; and somatic cell count > 500,000 cells/ml and positive to the CMT (> 2 CMT). Only cows with one quarter affected were included in this trial. This classification of macroscopically characteristics of milk and somatic cell counts, allow the establishment of inclusion parameters in clinical studies (Bradley, 2012; Smith, 2009; Savas et al, 2012).

Treatment of CL and Exp-L groups consisted of one intramammary application of a commercial available formulation of amoxicillin plus potassium clavulanate (300 mg and 60 mg, respectively), every 12 hours for 3 consecutive days. In groups Exp-EL1 and Exp-EL2, two and three intramammary administrations of the same commercial formulation and in the same period of time were applied, respectively. All cows were first milked as much as possible, then the mammary gland was washed, disinfected and dried with a clean paper-towel. The cannula was then inserted until halfway within the teat canal, the drug combination was delivered and finally a gentle massage was applied to the affected gland (Martínez-Cortés, 2013).

Milk samples from the treated quarter or affected and treated quarter (mammary gland) were obtained by manual milking into 50 ml plastic tube, every 12 h for 7 days after the last application of antibiotics and immediately frozen in liquid nitrogen for no more than 21 days.

Identification and quantification of amoxicillin was carried simultaneously by isocratic reversed phase high performance liquid chromatography (HPLC), with 228

Table 1. *Mean elimination half-life (T½β) and elimination hybrid rate constant (β) values for amoxicillin in dairy cow's milk after intramammary treatment with a commercial preparation of amoxicillin-potassium clavulanate (300 mg/60 mg), every 12 hours for three days. CL stands for healthy cows; Exp-L for cows affected with mastitis and treated as described; Exp-EL2 stands for cows treated under extra-label route condition, by doubling the referred dose (same dose interval) and Exp-EL3 by tripling the dose.*

Treatment	Parameter	
	T½β	β
CL	63.05[a]	1.96
Exp-L	73.53[b]	2.08
Exp-EL1	82.85[c]	1.96
Exp-EL2	83.69[c]	1.84

a,b,c Between groups, parameters with uncommon superscript differ at P < 0.05

nm UV detection as proposed by Mohsen et a., (2007). A minimum detection and quantification limits of 4 and 5 µg/ml were obtained. Briefly, milk samples were thawed and fat removed. Amoxicillin was methanol extracted after vortexing and centrifuging the samples at 3,000 g for 20 min at -4ºC. The clear supernatant was used for drug identification and quantification. A solid/liquid borosilicate cartridge was used as a pre-cleaner system. Drug separation was accomplished by a stationary reversed-phase Chromolith® Performance (RP-18e, 100 mm x 4.6 mm) column with a 0.02 M disodium hydrogen phosphate buffer-methanol pH 3.0 (96:4, v/v) as isocratic mobile phase. Ampicillin was used as internal standard.

Final drug concentration in milk was estimated considering: milk sample volume, solvent extractor volume, chromatograph volume sample injected, signal chromatogram size area, and similar retention time of sample signal to drug standard signal. Chromatogram size area of each sample was transformed to drug concentration based on a standard curve, and linear over the range of 0.05 to 40 µg/ml (correlation coefficient > 0.99). The within-run and between-run precision values, as well as recovery percentages for amoxicillin were ≤ 5, ≤ 6, and 92%, respectively.

Results are presented as mean values ± SE. Differences in amoxicillin concentrations between experimental groups (Exp-L, Exp-EL1 and Exp-EL2) and control cows (CL) were evaluated from 12-72 h sampling period by using two-way ANOVA, followed by Dunnett's multiple comparisons test. Time and treatment effect, along with its interaction were included in the model. Differences in the percentage of cows reaching amoxicillin MRL along time were detected by comparison of survival curves. To accomplish this, a Log-rank (Mantel-Cox) test was performed. Finally, in order to evaluate the ability of cows in each group to clear the antibiotic throughout time, experimental data were fitted into non-linear regression curves. For this, a Log (time) vs response (I) or normalized response (II) equations was used. The following Hill equations were used to fit experimental data:

Y= Bottom+ (Top-Bottom)/[1+10^((LogT1/2-X)*h)] (equation I)

Y= 100/[1+10^((LogT1/2-X)*h)] (equation II)

Where: X is the logarithm of time, Y is the response, decreasing as X increases (equation I) or the normalized concentration of amoxicillin, which goes from 100 to 0%, decreasing as X increases (equation II), Top and Bottom are plateaus in the same units as Y, LogT1/2 is the time causing

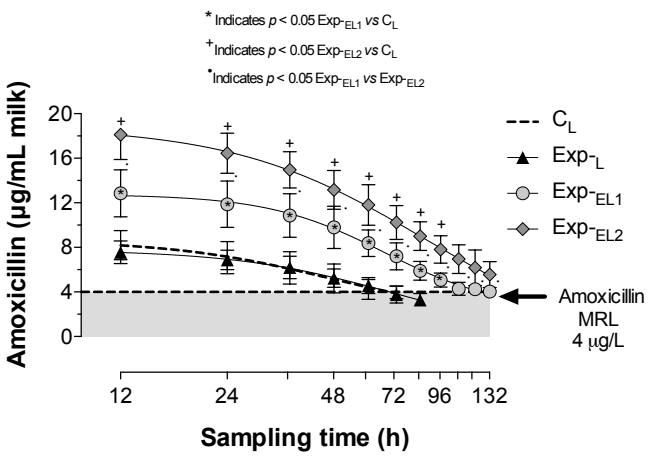
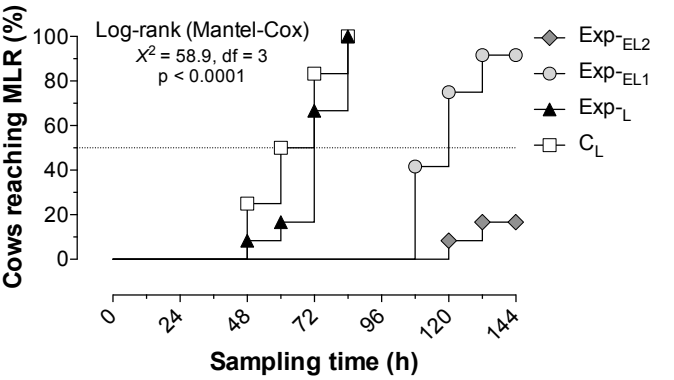


Figure 1. *Mean ± 1 SD elimination profiles of amoxicillin in milk from dairy cows after treatment with a commercially available preparation of amoxicillin-potassium clavulanate. Treatment was administered by the intramammary route, every 12 hours for three days. CL stands for healthy cows; Exp-L for cows affected with mastitis and treated as described; Exp-EL2 stands for cows treated under extra-label condition by doubling the referred dose (same dose interval) and Exp-EL3 by tripling the dose. The accepted MRL for amoxicillin in milk is marked in grey. Different signs between curves stand out for differences statistically significant.*

50% reduction of the initial concentration and β is the hybrid elimination rate constant. For this analysis, amoxicillin concentration was normalized to 100%, which corresponded to the initial value during the assessed period of time. From the obtained parameters of each curve, the extra sum-of-squares F-test was used to compare elimination T½ between groups. Official MRL adopted for this trial was 4 µg/ml (EMEA, 2001; Samanidous et al., 2007; Kneebone et al., 2010).

In all cases, the probability level considered as significant was $P < 0.05$. All statistical analysis and graphs were generated on Prism 6.0 (GraphPad Software, Inc., USA).

Figure 2. *Survival analysis that shows the number of cows that reached the desired concentration of amoxicillin (below the MRL of 4 µg/L) after treatment with a commercially available intrammamary preparation of amoxicillin-potassium clavulanate (300 mg/60 mg), every 12 hours for three days.*



RESULTS

Figure 1 shows depletion profiles of amoxicillin in cows from all four treatment groups. Values of elimination half-life (T½β) and the hybrid rate constant for elimination (β) increased after double- and triple-dose mastitis therapy, showing a zero-order tendency. Changes in these values were statistically significant ($P < 0.05$). A clear and statistically significant difference was observed in elimination kinetics of amoxicillin, when comparing T½β and β values of CL and Exp-L groups against Exp-EL2 and Exp-EL3 groups. Also, a statistically significant difference was obtained when Exp-EL2 and Exp-EL3 were compared (see Table 1). By means of a survival analysis, it becomes clear that amoxicillin is cleared beyond 6 milking times or 72 h, claimed by the manufacturer in CL and Exp-L groups. In contrast, Exp-EL1 and Exp-EL2 groups required more than 132 h to reach the desired MRL. In Figure 2, the survival analysis applied to withdrawal times shows the number of cows that reached the desired concentration of amoxicillin below the MRL of 4 µg/L.

DISCUSSION

Withdrawal times for milk are established based on approved analytical methods, mainly through high performance liquid chromatography and after studies of population pharmacokinetics. Thus, minimum deviations from the mean are expected when data is applied under field conditions. In this trial, the use of a validated analytical technique (Mohsen et al, 2007) rendered recovery values, as well as intra and inter-assay errors that can be regarded as reliable. However, large standard deviation values were obtained. These are clearly the results of the inherent variability of clinical cases of mastitis, as well as the milk yield of each cow. In this respect, results obtained in this trial stand out as noticeably different from residue studies carried out by sponsors of a given pharmaceutical product, where healthy and more homogeneous types of animals are chosen to avoid dispersion of data (Jones et a., 1999). In spite of the above, withdrawal time established in this study comply with the one proposed by the pharmaceutical company for its product. That is, variability of milk producing cows in CL group with healthy cows and cows affected with mastitis in Exp-L group, showed that elimination

of amoxicillin in these two breeds of cows is essentially the same. This is not the case for other drugs such as cefoperazone after its intramammary infusion (Cagnardi et al, 2010), but is in agreement with cefquinome tested under similar conditions and where persistence of the drug was similar between healthy and infected cows (Zonca et al., 2010).

In contrast with the above, these results suggest that if clinical interpretation of a case ends up in a decision of doubling or tripling the recommended dose, an uncertain extension of the withdrawal period is expected. This is especially true in cases such as this, where amoxicillin elimination showed a zero-order kinetics. There are few studies in literature that could validate or contradict that the extra-label maneuver of doubling or tripling the recommended dose helps to improve the outcome of a given case of mastitis (Gruet et al, 2001).

Additionally, it can be stated that among clinicians, there is not always sufficient awareness on the effect that this decision can have on the occurrence of illegal residues in milk. Increments in withdrawal time in this study reached 48 h. That is, four milkings, one every 12 h, when the recommended intramammary dose of amoxicillin-potassium clavulanate was triplicated. Important variations in clearance of antibacterial drugs should also be expected in cows with less milk yield among the cows treated (Stockler et al, 2009). An additional issue is the rationale for using double or triple dose of antimicrobial products. The most important factor, in successful β-lactamic antibiotic therapy, is the length of time that the drug concentration in milk and mammary tissue remains above the minimum inhibitory concentration for the causative bacteria and not how much higher the maximum drug concentration achieves over the minimum inhibitory concentration.

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