

Withdrawal of amoxicillin and penicillin G procaine from milk after intramammary administration in dairy cows with mastitis

Artur Burmańczyk¹, Grabowski Tomasz², Małgorzata Gbylik-Sikorska³,
Anna Gajda³, Cezary Kowalski¹

¹Department of Pharmacology, Faculty of Veterinary Medicine,
University of Life Sciences, 20-033 Lublin, Poland,

² Polpharma Biologics, 80-172 Gdańsk, Poland

³ Department of Pharmacology and Toxicology, National Veterinary Research Institute, 24-100 Pulawy,
artur.burmanczuk@up.lublin.pl

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Abstract

Introduction: There are many veterinary products containing β -lactam antibiotics which are used for mastitis treatment in cows. The aim of the study was to determine whether mastitis could have any effect on amoxicillin (AMX) or penicillin G procaine (PEN) withdrawal period from milk, in the context of current maximum residue limits established by the European Commission. **Material and Methods:** The study was conducted on 17 dairy Black and White cows with clinical mastitis during the lactation period. The first group ($n = 8$) received 200 mg of amoxicillin (AMX), whereas the second group ($n = 9$) received 200,000 IU/mg of penicillin G procaine (PEN) by intramammary administration. For the measurement of AMX and PEN concentrations in milk, the liquid chromatography tandem mass spectrometry method was applied. Pharmacokinetic calculations were performed using Phoenix WinNonlin 6.4 software. **Results:** The determined AMX and PEN half-life values in the mammary gland suggest that the drug withdrawal is at a level of 99.9% within 81 h (≈ 3.5 days) and 116 h (≈ 5 days) after administration of AMX and PEN, respectively. The present research indicates that, at 60 h after administration, the average PEN concentration in the milk from cows with clinical signs of mastitis may still reach 4.96 g/kg and that of AMX can even be 6.92 g/kg. **Conclusion:** The results obtained confirm that, in mastitis cases, a 72-h withdrawal period is sufficient for elimination of AMX to a lower level than the established maximum residue limit (MRL) values. However, in the case of PEN, at 69 h after administration, the drug concentration may be close to that of the determined MRL.

Keywords: cows, milk, amoxicillin, penicillin G procaine, pharmacokinetics.

Introduction

According to the European Commission (EC) report, which presents a medium-term outlook for the major European Union (EU) agricultural commodity markets and agricultural income to 2024, the expansion of EU milk production is limited by increasing levels of production in competing regions (17). However, the large-scale production in some EU countries is associated with great responsibility and the need for the best practice to maintain quality (17). Inflammation of the mammary gland in cows is an important sanitary, epidemiological, and economical issue. Introduction of therapeutic agents into udder quarters, emptied from inflammatory secretions, if not accompanied by

inflammation of the overall reaction system, is a principle in the treatment of mastitis. There are a number of veterinary products containing β -lactam antibiotics in their composition, which are used for mastitis treatment in cows (4, 6, 9, 24). One of the β -lactam antibiotic groups are aminopenicillins, including penicillin G procaine (PEN) and amoxicillin (AMX) (6, 25). Penicillins are commonly used in veterinary medicine due to their broad spectrum of activity when confronted with natural penicillins, low treatment cost and safety (24, 25). When collated with penicillins G, aminopenicillin has a better ability of penetration of the external cell membrane layer and, consequently, it has a much broader spectrum of activity against many Gram negative bacteria (e.g.

Enterobacteriaceae spp.) (24). A severe limitation in their therapeutic use is the increasing resistance of bacteria to PEN and AMX (6, 25, 30, 33).

Uncontrolled use of aminopenicillins and penicillins in animal treatment may result in the presence of antibiotic residues in food of animal origin (6, 27, 30, 31). However, the risk to human health is posed by antibiotic residues in milk produced by cattle treated for mammary gland inflammation (6, 22). Due to the potential impact on human health, the EC established maximum residue limits (MRLs) for PEN and AMX in milk at the level of 4 µg/kg (11). For this reason, pharmacokinetic studies with aminopenicillin and penicillin were conducted in order to determine their withdrawal periods in milk. It should be noted that determination of withdrawal periods and MRL values does not involve comparative analysis and parallel or cross-over examinations of healthy and diseased animals. The withdrawal period is established exclusively in a group of healthy animals (10).

The aim of the study was to determine whether mastitis could have any effect on AMX or PEN withdrawal from the cow milk. The study involved cows with clinical symptoms of mastitis receiving intramammary doses of the analysed drugs. The determination of the concentration of AMX and PEN in milk was performed by a validated and selective liquid chromatography tandem mass spectrometry method.

Material and Methods

The study was conducted on 17 dairy cows of the Polish Black-White breed at the age of 4–10 years with clinical mastitis during the lactation period. The cows were examined in different lactation periods, *i.e.* from 1.5 month post-calving to the pre-drying period; the average milk yield per cow was ca. 25–30 L/24 h. The cows weighed approx. 650 kg each. They were qualified for the research on the basis of guidance from the owners and research exchanges (12). According to the EMA guidelines, subclinical mastitis is diagnosed if somatic cell count (SCC) is over 200,000 cells/mL (12). The cows were verified using a mastitis detector DRAMINSKI Mastitis Detector 4 × 4Q, a 4-quarter device for detecting subclinical mastitis in its earliest, visually undetectable stage (Dramiński, Poland), in order to assess the SCC. Cows with $SCC \geq 400,000/\text{mL}$ were qualified for the study. The animals were fed farming feed concentrates comprising commercial (wheat, rye) and fodder (oats, barley) grain alternating with raw corn and pasture grazing. Access to feed and water was provided *ad libitum*. The analyses were carried out between March and September. The cows originated from different farms in the Lublin Province area. The first group of cows ($n = 8$) received a 5 mL water solution containing 200 mg of AMX (Synulox L.C., Pfizer, Poland), whereas the second group ($n = 7$) received a 5 mL water solution containing 200,000 IU/mg of PEN (Albadry Plus, Zoetis, Poland) by intramuscular

administration. PEN and AMX were used as indicated by the manufacturer's label. Before the process of milk sample collection, the teats were cleaned and immersed in a liquid disinfectant, Avitaderm (Agrovet, Poland). Afterwards, their mouths were wiped with a swab soaked in 70% ethanol. Subsequently, after single intramuscular administration, the milk samples (10 mL) were collected at the following time points: PEN after 2, 4, 6, 8, 10, 24, 36, 48, and 60 h and AMX after 2, 4, 6, 8, 10, 24, 36, 48, and 72 h. The milk samples were collected into dark plastic bottles and kept in a cooler at -18°C until analysis.

For the measurement of AMX and PEN concentrations in milk, LC-MS/MS method was applied, based on a previously described procedure for determination of antibiotics in muscle tissues, including AMX and PEN (2). The extraction of AMX and PEN with acetonitrile established for muscles was found suitable for the isolation of these two compounds from the milk matrix. Sample preparation procedure for AMX and PEN in milk was the same as the procedure used for muscle tissue. The only difference between the analysis described by Błądek *et al.* (2) and the method used in the presented study was the omission of the homogenisation step before the extraction. Two grams of milk were directly weighed into a polypropylene centrifuge tube and 8 mL of acetonitrile was added in the extraction step. The same LC-MS/MS conditions and analytical equipment were used. The chromatographic separation of AMX and PEN performed on the Luna[®] octadecyl C18 column 150×2.0 mm, 3 µm with the mobile phase consisting of acetonitrile and 0.025% heptafluorobutyric acid was also suitable for the milk samples. For the purposes of the conducted study, the whole validation procedure for the milk matrix was performed. The method used in the presented experiment was fully validated in accordance to Commission Decision 2002/657/EC (16). During the validation process, various parameters were investigated, such as linearity, precision (repeatability and within-laboratory reproducibility), accuracy, decision limit (CC_{α}), and detection capability (CC_{β}). Additionally, the limit of detection (LOD) and the limit of quantification (LOQ) were established. For linearity, a matrix-matched calibration curve was prepared in duplicate at the range of 2–10,000 µg/kg. The validation results showed acceptable linear response. The correlation coefficients for AMX and PEN were over $R > 0.99$. Precision was determined by the repeated analysis ($n = 6$) of milk samples spiked with analytes at concentrations corresponding to $0.5 \times \text{MRL}$, MRL, and $1.5 \times \text{MRL}$, between runs during one and three days, respectively. The validation results obtained for the presented method were repeatable and reproducible with the repeatability calculated as a percentage of relative standard deviation (RSD%) lower than 15% at all fortification levels and within-laboratory reproducibility lower than 20%. The overall coefficient of variations (CVs) for both analytes was in the range of 9.8%–13.6% for repeatability and 9.2%–15.8% for within-laboratory reproducibility. The

validation results showed acceptable accuracy with the average recoveries ranging from 93.3% to 96.4% for AMX and from 93.2% to 95.5% for PEN. The CC_{α} and CC_{β} for AMX were 5 $\mu\text{g/kg}$ and 7 $\mu\text{g/kg}$, respectively. The CC_{α} and CC_{β} of PEN were established at 4 $\mu\text{g/kg}$ and 6 $\mu\text{g/kg}$, respectively. The procedure was satisfactorily sensitive with $\text{LOD} = 1 \mu\text{g/kg}$ and $\text{LOQ} = 2 \mu\text{g/kg}$ for both analytes.

Pharmacokinetic and statistical analysis. The pharmacokinetic calculations were performed based on raw data using Phoenix[®] WinNonlin[®] 6.4 software (Certara L.P., USA) and the statistical analyses were carried out with GraphPad Prism[®] 6.01 programme (GraphPad Software Inc., USA). The calculations were based on the slope, height, area, and moment (SHAM) analysis, and a modified two-compartment model of drug distribution. The following parameters were calculated with the SHAM analysis: AUC_{0-t} – area under the curve calculated between zero and the last sampling point; $\text{AUC}_{0-\infty}$ – area under the curve calculated between zero and infinity; AUMC_{0-t} – area under the first moment curve calculated between zero and the last sampling point; $\text{AUMC}_{0-\infty}$ – area under the first moment curve calculated between zero and infinity; MRT_{0-t} – mean residence time calculated for the last sampling point; $\text{MRT}_{0-\infty}$ – mean residence time calculated for infinity; $\text{AUC}_{\text{rest}\%}$ – percentage of the rest area under the curve; C_{max} – maximal concentration; t_{max} – time to reaching maximal concentration; C_{last} – last measured concentration; t_{last} – time of the last measured concentration; k_{el} – elimination rate constant. As part of the compartment analysis, the following were additionally calculated: k_d – distribution rate constant; $t_{1/2k_{\text{el}}}$ – elimination half-life; $t_{1/2k_d}$ – distribution half-life; B – concentration extrapolated by elimination phase; A – concentration extrapolated by distribution phase; k_{10} – elimination rate from milk (central) compartment; k_{20} – elimination rate from tissue compartment; k_{12} – rate constant between milk and tissue concentration; k_{21} – rate constant between tissue and milk concentration; CL – total clearance; V_1 – milk compartment volume; V_2 – tissue compartment.

The compartment analysis distinguished two compartments. The first one was the central compartment comprising milk present in the teat canal and teat sinuses. Udder tissues were defined as the second compartment. Therefore, the distribution rate constant k_d illustrates the elimination rate constant for drug withdrawal with milk. Hence, the equation for calculation of the AMX and PEN concentrations at any time point (C_p) following intramuscular administration has a typical structure of the two-compartment model:

$$C_p = A \times e^{-k_d \times t} + B \times e^{-k_{\text{el}} \times t} \quad (\text{Eq. 1})$$

In turn, the elimination rate constant k_{el} represents drug transfer from milk to tissues. Between-animal variability was shown as percentage of relative standard deviation (RSD). A *t*-student test was employed to confirm the significance of these observations. Differences with a $P < 0.05$ were considered significant.

Results

The results of chromatographic measurements of AMX and PEN in cow's milk after intramuscular administration are shown in Table 1 and Figs 1A and 1B. An immediate maximal concentration of the drugs in plasma can be observed after intramuscular administration. Next, it decreases quickly as a result of distribution with milk into the tissue compartment. The difference in the chemical structure in the key parameters between the studied drugs is not significant. Lipophilicity (XlogP), topological polar surface area, hydrogen bond donors, and hydrogen bond acceptors in the case of AMX and PEN are -2, 158 \AA^2 , 4, 7 and 1.8 (penicillin G), 169 \AA^2 , 4, 10, respectively

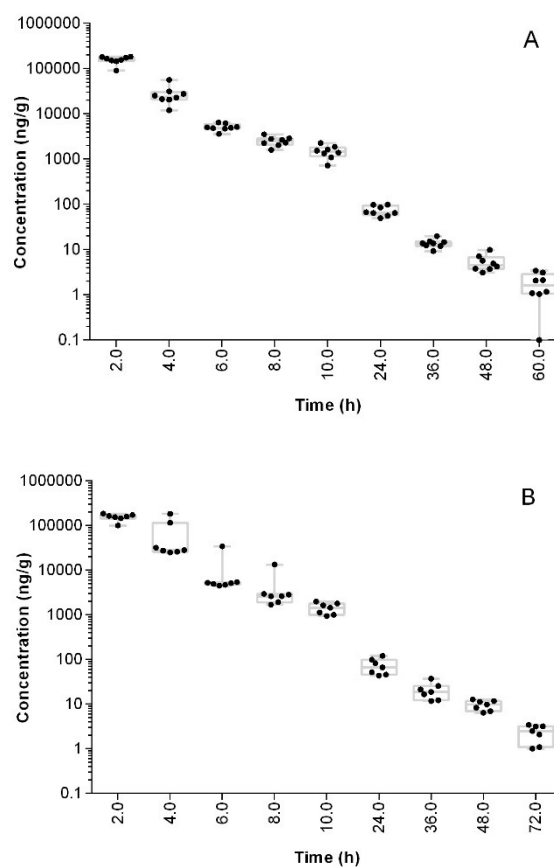


Fig. 1. Kinetics of withdrawal of amoxicillin (A) and penicillin G procaine (B) after a single intramammary administration of the drug at a dose of 200 mg and 200,000 IU/mg, respectively

The results of the pharmacokinetic analysis are presented in Table 1. The calculations indicate that PEN in the intramammary formulation can be classified as a drug with high individual variability. This variability in the case of PEN in relation to AUC_{0-t} was $>30\%$. The same parameter in the case of AMX was only 18%. However, in the case of C_{max} , both AMX and PEN exhibited low values of individual variability, *i.e.* 18% and 17%, respectively. It should be noted that the individual variability in the case of the other analysed parameters was low and at a similar level (AMX vs. PEN),

Table 1. Pharmacokinetic parameters describing the behaviour of the amoxicillin and penicillin G procaine after a single intramammary administration of the drug at a dose of 200 mg and 200,000 IU/mg, respectively

PK parameters	Unit	Amoxicillin		Penicillin G procaine	
		M	SD	M	SD
AUC _{0-t}	g×h/kg	391974.52	69043.63	471040.97	146203.3
AUC _{0-inf}	g×h/kg	391998.61	69045.23	471080.73	146209.5
AUMC _{0-t}	g×h ² /kg	1087333.96	207114.07	1441652.85	588232.58
AUMC _{0-inf}	g×h ² /kg	1089094.32	207140.19	1445211.84	588599.82
AUC _{rest%}	%	0.01	0.0	0.01	0.0
MRT _{0-t}	h	2.77	0.13	2.99	0.3
MRT _{0-inf}	h	2.78	0.13	2.99	0.3
k _{el}	1/h	0.088*	0.016	0.061*	0.008
k _d	1/h	0.805	0.106	0.857	0.118
B	g/kg	403.45	242.04	191.73	84.11
A	g/kg	747130.27	276354.99	1911448.46	2530672.32
t _{1/2kel}	h	8.11*	1.55	11.61*	1.68
t _{1/2kd}	h	0.88	0.13	0.82	0.12
C _{max}	g/kg	154850.0	27867.68	156571.43	27191.69
t _{max}	h	2.0	0.0	2.29	0.7
C _{last}	g/kg	1.75	1.07	2.35	0.93
t _{last}	g/kg	60.0	0.0	72.0	0.0
k ₁₀	1/h	0.802	0.107	0.854	0.117
k ₂₁	1/h	0.089*	0.017	0.061*	0.008
k ₁₂	1/h	0.003	0.001	0.002	0.001
k ₂₀	1/h	25.26	7.368	44.81	40.905
CL	L	0.535	0.143	0.467	0.143
V ₁	kg	0.344	0.214	0.212	0.102
V ₂	kg	10.08	5.32	8.368	3.584

M – arithmetic mean; SD – standard deviation; AUC_{0-t} – area under the curve calculated between zero and last sampling point; AUC_{0-inf} – area under the curve calculated between zero and infinity; AUMC_{0-t} – area under the first moment of curve calculated between zero and last sampling point; AUMC_{0-inf} – area under the first moment of curve calculated between zero and infinity; MRT_{0-t} – mean residence time calculated for last sampling point; MRT_{0-inf} – mean residence time calculated for infinity; AUC_{rest%} – percentage of the rest area under the curve; C_{max} – maximal concentration; t_{max} – time to reach maximal concentration; C_{last} – last measured concentration; t_{last} – time of last measured concentration; k_{el} – elimination rate constant; k_d – distribution rate constant; t_{1/2kel} – elimination half-life; t_{1/2kd} – distribution half-life; B – concentration extrapolated by elimination phase; A – concentration extrapolated by distribution phase; k₁₀ – elimination rate from milk compartment; k₂₀ – elimination rate from tissue compartment; k₁₂ – rate constant between milk and tissue concentration; k₂₁ – rate constant between tissue and milk concentration; CL – total clearance; V₁ – milk compartment volume; V₂ – tissue compartment; * – P < 0.05

but significant differences were found in some cases. The variability of the k₁₂ and k₂₀ values (RSD ≈ 67%) was two- and three-fold higher, respectively, than that in the case of PEN. The variability of A (RSD ≈ 132%) was 3.6-fold higher than that of PEN. The proposed two-compartment model was verified by the calculations. The k_d/k_{el} value for AMX and PEN was 9.1 and 14.1, respectively. This indicates that processes referred to as distribution reached 9- and 14-fold higher rates than the determined k_{el}. The t_{1/2kel} calculated on the basis of MRT_{0-t} was 4.1- and 5.5-fold shorter than that calculated based on k_{el}. Sampling was carried out at 7.4 and 6.2 half-lives after administration of AMX and PEN, respectively. This allowed the maintenance of the minimal values of the residue area at a very low level (Table 1). The determined AMX and PEN half-life values in the mammary gland suggest AMX and PEN withdrawal at a level of 99.9% (10×t_{1/2kel}) within the time of 81 h (≈3.5 days) and 116 h (≈5 days) after administration of the drug, respectively. The kinetics of both drugs, particularly in terms of the analysis of rate constants, was highly similar. Significant differences were only evident in the case of k_{el}, t_{1/2kel}, and k₂₁ (Table 1).

Discussion

Pharmacokinetic profiles of AMX and AMP in animals have been presented in several publications (7, 8, 9, 14, 15, 20, 21, 29). Ampicillin has been studied in alpacas (28), llamas (5, 29), lactating sheep and goats (15), cattle (12), female buffaloes (26), and neonatal calves (21). The pharmacokinetic profile of AMX has been investigated in sheep and goats (7, 8, 9, 14, 20). However, up to now, there has been no detailed research involving AMX and PEN pharmacokinetics in milk after IM administration to cows of the Black and White breed showing signs of clinical mastitis.

In the present study, after intramuscular administration of AMX and PEN, both substances were detectable with the use of LC-MS/MS in milk for 60–72 h after single drug administration. Parenterally administered acidic antibiotics are quickly eliminated from milk (6, 9, 23, 24). After intravenous administration, the AMX and PEN elimination half-lives are 7.5 to 11.8 h, respectively (1, 14, 21, 32). Furthermore, quick AMX elimination from milk (48 h) was observed after intravenous administration in llamas (29).

The above-mentioned results of the sustenance time of AMX and PEN in milk after intravenous administration indicate limited suitability of the parenteral use of these antibiotics in mastitis treatment. A high rate of elimination with a large decrease in the concentration of AMX and PEN was recorded within 12 h after intramuscular administration of the drugs. In the study conducted by Bruno *et al.* (3) milk samples were taken after intramuscular administration of 5 g of AMX ($n = 1$). Three days later the AMX concentration was 2.7 g/L and after six days 1.2 g/L. This means that the half-life in this phase was ≈ 61 h. The present research was carried out on cows of Black and White breed at $C_{\text{last}} = 72$ h; hence, it was difficult to verify the AMX half-life in the period between days 3 and 6 after administration of the drug. The investigations reported by Bruno *et al.* (3) were performed on milk from one cow only. The breed of the cow from which milk was sampled was not specified ($n = 1$). However, the results obtained by the authors indicate fluctuating or non-linear characteristics of AMX elimination.

Undoubtedly, the pharmacokinetic profiles of AMX and PEN are highly similar. The differences observed are definitely negligible and mostly statistically insignificant ($P > 0.05$). An interesting element of the AMX and PEN kinetics is the fact that both drugs administered *via* the intramuscular route are characterised by typical two-compartment kinetics. The distribution of both drugs to the tissue compartment is definitely a separate kinetic phase. This implies that the transfer from tissues into milk and from udder tissues to systemic circulation is an important element of elimination of the drugs. However, the V_2 value seems negligible. The results of the analyses indicate that it ranges from 5 to 15 kg of tissue. Therefore, a hypothesis can be proposed that elimination of the drugs proceeds in udder tissues and they hardly reach systemic circulation. The k_d in the adopted model is of particular importance. One of the elements of distribution is drug withdrawal from milk; however, the high dynamics of this phase is maintained in both cases only until ca. 12 h after administration of the drugs. Hence, AMX and PEN elimination is not dependent on production of milk by the mammary gland only. During the subsequent post-administration hours, the concentration curve slope is remarkably milder and drug elimination is mainly dependent on the mutual ratio of the k_{12} , k_{21} , and k_{10} rate constants. In the case of both drugs, k_{20} has very high values, which means that the drug is eliminated from the tissue compartment at a very high rate. Assuming that k_{10} denotes a drug withdrawn from milk, the rate of AMX and PEN elimination from tissues is 31- and 52-fold higher, respectively, than the rate of drug withdrawal from milk. In both cases, however, the drug transfer to tissues is a relatively slow process. This is indicated by the k_{21}/k_{12} ratio, which shows that the AMX and PEN transfer from milk to tissues is 30 and 33 times slower than that from tissues to milk. This fact explains

e.g. the very fast rate of elimination of both drugs from the tissue compartment (k_{20}).

The calculations reveal that at 60 h after a single intramuscular administration, the AMX concentration in milk is 2.0 g/kg. Notably, the individual variability of this time point in the case of AMX reaches 61%. This means that, at 60 h after administration, the drug concentration may reach a level of even 3.22 g/kg. It should be noted that in the USA, France, and Portugal, the withdrawal period for milk from cows treated with intramammary AMX formulations is only 24 h (12, 18). However, the presented calculations show that the AMX concentration in milk from Black and White breed cows with signs of clinical mastitis at 24 h after administration of a dose of 200 g of the drug can have an even 12-fold higher mean value (>48 g/kg) than MRL (4 mg/kg).

After a single intramuscular administration, PEN concentration calculated using Eq. 1 after 69 h is 2.87 g/kg. The individual variability for this time point in the case of PEN is 39%. Therefore, 69 h after PEN administration the concentration of the drug may still exhibit a level of 3.99 g/kg, *i.e.* a value close to MRL. At the variability mentioned above, PEN concentration equal to $0.5 \times \text{MRL}$ can be achieved at 80 h after administration of the drug. The results obtained confirm that in mastitis cases, a 72-h withdrawal period is sufficient for elimination of AMX to a lower level than the established MRL values. However, in the case of PEN, at 69 h after administration the drug concentration may be close to that of the determined MRL. This implies that, depending on the individual variability, differences among breeds, variation in nutrition, or changes in the udder blood flow, the threshold can be exceeded. It should be added that the PEN withdrawal period was established in the USA at 60 h for lactating cows and 72 h for cows in the dry period (19). The present study, however, demonstrates that a 60-h post-administration period in the case of PEN is not sufficient to reach concentrations corresponding to the standards established by the EC. The present research indicates that at 60 h after administration, the average PEN concentration of the drug in the milk from Black and White breed cows with clinical signs of mastitis may reach 4.96 g/kg with 39% between-animal variability. In the case of AMX, mastitis has no significant impact on the withdrawal period and the safety of application of intramammary formulations. Therefore, the presented results are consistent with those obtained in investigations on healthy cows, which were the basis for recording and establishment of the withdrawal period for the original formulation (10).

Guidelines regulating aspects related to the calculation of MRLs for drugs administered *via* the intramuscular route emphasise the role of selection of cattle intended for investigations (13). Regulatory authorities attach a significant role to the impact of the cattle breed on the rate of drug withdrawal in milk. The present study was carried out on the Black and White

breed, for which the kinetics of AMX and PEN elimination in the case of mastitis has not been described yet.

The lack of harmonised requirements for the levels of AMX and PEN residues in milk at the level of the International Conference of Harmonisation still impedes uniform control of the residues of these drugs in milk at a global scale. In the area of EMA influences, the admissible level of AMX and PEN residues is 4 g/kg; in turn, in regions influenced by the FDA, the values are 10 g/kg (AMX) and 0 g/kg (PEN) (18, 19). There are no regulations concerning the cattle breed that can be used in investigations of drug residues and regarded as a representative breed. Neither are there regulations for elimination periods for drugs with kinetics partly dependent on blood flow through the mammary gland. Blood flow can be influenced not only by the form and course of mastitis, but also by individual differences or differences related to breed, and lactation phase, and consequently those related to the cardiac output. These issues have still not been defined by relevant guidelines. The present study indicates that there are still real potential risks for consumers and milk processing industry.

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