Full title: Determination of the intramammary dose of benzylpenicillin required to maintain an adequate concentration in the milk to inhibit Gram-positive bacteria in the clinically normal udder for 24 hours.

Short title: Determination of the intramammary dose of penicillin

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Abstract

The aim of this study was to determine the intramammary dose of benzylpenicillin required to maintain a concentration in the milk above the MIC for the Gram-positive bacteria that cause mastitis. The product used in this study was a commercially available procaine benzylpenicillin in an oily suspension with micronized particles. Three dose levels were used: 200 000, 300 000 and 600 000 IU. Concentrations of benzylpenicillin in cow milk and plasma were determined after a single intramammary dose was administered into one quarter of each of the 5 cows in each treatment group. Samples were analyzed using an HPLC-MS/MS method which was validated during the study. Concentrations in the milk were well above the MIC for the target pathogens for all doses tested. There was a linear dose-dependent increase in the mean AUCs of benzylpenicillin concentrations in plasma and milk. At the first milking, 12 hours after dosing, there was a significant difference between the mean milk benzylpenicillin concentrations in cows treated with a dose of 600 000 IU, and

those treated with 200 000 or 300 000 IU. Although this study shows a linear relationship between the dose of procaine benzylpenicillin administered and the concentration in the milk in the healthy udder, it would be useful to conduct studies on cows with mastitis to define the optimum dose and duration of intramammary treatment with benzylpenicillin.

Keywords: penicillin, intramammary, clinical mastitis, Gram-positive bacteria, β-lactam antibiotics

Introduction

Antimicrobial resistance is an emerging issue, so the use of these drugs in food-producing animals is being scrutinized worldwide (Laxminarayan, Duse et al., 2013). Globally, the most important indication for antibiotic use on dairy farms is mastitis (Pol & Ruegg, 2007; Stevens, Piepers et al., 2016). Benzylpenicillin is considered to be the first-line treatment for mastitis caused by Gram-positive bacteria because of its narrow spectrum, according to the European Union's responsible use guidelines (European_Commission, 2015). Benzylpenicillin is a β-lactam antibiotic with bactericidal action against the major Gram-positive bacteria often found in mastitis, such as *Streptococcus* spp. and *Staphylococcus* spp. (Prescott, 2013). All β-lactam antibiotics have a time-dependent mode of action, meaning that treatment success mainly depends on the length of time that the drug remains above the MIC (Turnidge, 1998; Erskine, Wagner et al., 2003; Prescott, 2013)

Intramammary infusion is the most common route of administration for the antibiotics used to treat mastitis (Gruet, Maincent et al., 2001). The aim of this treatment is to target the actual infection site by administering the antibiotic into the udder, and for the concentration of the drug to reach levels above the MIC for the bacteria causing the infection. The active ingredient must not only distribute well into the milk phase inside the udder, but also be absorbed into the tissue if the infective agent is an invasive (and potentially intracellular)

pathogen, such as *Staphylococcus aureus* (Ziv, 1980a). The distribution of penicillin administered via the intramammary route was investigated in an isolated perfused model by Ehinger and Kietzmann (2000). Penicillin in oily suspension with small particles (micronized form) was found to have the best glandular distribution compared to aqueous solution or oily suspension with large particles. Absorption into the perfusate was also greatest after treatment with the oily suspension with small particles, delivering the highest areas under the absorption-time curves (Ehinger & Kietzmann, 2000).

As a result, the decision was taken to use benzylpenicillin in oily suspension with small particle size to formulate a new intramammary penicillin product. According to the European Medicines Agency (EMA) guidelines on the conduct of pharmacokinetic studies in target animal species, the dose of the active substance needs to be determined using the concentration of penicillin in the milk as a function of time to allow the therapeutic concentration-time profile at the infection site in the udder to be estimated. The same procedure should be applied to the plasma to estimate potential systemic absorption. Dose determination studies usually require three different dose levels to be tested and are typically performed in healthy animals (EMA, 2000).

The aim of this study was to determine the intramammary dose of benzylpenicillin required to maintain a concentration in the milk above the MIC for 24 hours for Gram-positive mastitis pathogens. Concentrations of benzylpenicillin in cow milk and plasma were measured following a single intramammary administration of benzylpenicillin procaine at three dose levels (200 000, 300 000 and 600 000 IU) into one udder quarter. The study was conducted in 2005 and followed the principles of the EMA guidelines for the conduct of pharmacokinetic studies in target animal species that were in force at the time (EMA, 2000) . The product was originally authorized for use in only a few countries, but in 2017

an identical copy of it was approved for use in fourteen countries of the European Union through the decentralized procedure.

Materials and Methods

Subjects and product administration

Fifteen clinically healthy, 2 to 5-year-old Holstein cows were selected for this study. The cows were between the 3rd and 6th month of lactation and had not received any antibiotic treatment in the 8 days preceding the trial. The average daily milk yield was 32 liters (range 22.9–43.5) at the beginning of the study. All animals were housed on the farm of origin, which was also the site of the study, but an acclimation period of 7 days was applied to allow pre-trial observation of the cows. Each cow was examined clinically once daily throughout the entire acclimation and study phase: udders were inspected and palpated, and milk was checked for the appearance of clots or flakes, and changes in color and consistency. Milk production was recorded at each milking. During the acclimation period, the cows were ranked by lactation stage, then randomly allocated into five lactation stage groups, each containing three animals, to ensure similar distribution of days in milk across the groups. Computerized random number generation was performed with Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA) to randomly allocate one animal out of each lactation stage group to each of three treatment groups. More details on the animals involved in the study can be found in Table 1.

Include Table 1 here.

The test product was a registered intramammary suspension (Carepen®, Vetcare Oy, Mantsala, Finland, also registered as Ubropen®, Boehringer Ingelheim Animal Health, Germany) containing 600 000 IU (equivalent to 600 mg) of micronized procaine

benzylpenicillin per 10 mL in an oily suspension. The product was administered intramammarily into the left front quarter. All animals were treated once after the morning milking on Day 0. For Group 1 (200 000 IU penicillin) and Group 2 (300 000 IU penicillin), two-thirds and one half, respectively, of the contents of a Carepen® /Ubropen® tube was discarded before administration to obtain the correct dose of benzylpenicillin procaine per cow. The full contents of the Carepen®/Ubropen® tube was administered to animals in Group 3. Tubes were weighed before and after administration to determine the actual dose given. Teats were properly cleaned with individual antiseptic wipes before administration.

The study was carried out in compliance with French legislation on the protection of laboratory animals, and in accordance with a valid license for experiments on vertebrate animals issued by the French Ministry for Agriculture. The study passed the ethical review committee of Avogadro (Fontenilles, France), the contract research organization that was responsible for the trial (Reference code of the study: *A051143*).

Milk and blood sampling

Before dosing, mixed milk samples were collected from all cows on day 0 from the milk obtained during regular milking. On day 1, milk samples from treated quarters were collected at 12, 24, 48 and 72 hours after dosing. The foremilk from the treated quarter was discarded and the teat was thoroughly cleaned and disinfected before each sample was collected. Three samples of about 5 mL of milk were collected from each treated quarter at each sampling session. The cow's total milk yield was also recorded at each sampling session. Samples were transported to the analytical laboratory (Avogadro, Fontenilles, France) at about 4 °C and frozen (to about -20 °C) as soon as possible.

Blood samples were collected from the jugular or tail vein before treatment and at 3, 6, 12, 15, 18 and 24 hours after administration. Blood (10 mL) was collected in lithium heparin tubes which were kept at 4 °C until centrifugation. They were centrifuged at around 2500 g for 10 minutes at 4 °C and 1.5 mL of plasma from each sample was transferred to each of 3 propylene tubes.

Quantification of benzylpenicillin

Samples were processed by the laboratory within 5 days of arrival. A stability test was performed, indicating that benzylpenicillin was stable in plasma at -20 °C for at least 9 days in storage (max. 20% degradation). Samples were analyzed by HPLC-MS/MS after precipitation of the protein with acetonitrile and solid-phase extraction with an OASIS® HLB cartridge (Waters Corporation, Milford, MA, USA). The Lower Limit of Quantification (LLOQ) of the testing method in milk and plasma was 2 ng/mL. For comparison, the Maximum Residue Limit (MRL) in the European Union is set at 4 µg/kg (or 4 ng/g) for milk (EMA, 1999). Specificity against endogenous substances was evaluated by carefully assessing the ion chromatograms from plasma from control cows at the retention times of benzylpenicillin and phenoxymethylpenicillin (the latter being the internal standard). No interfering peaks were observed at these times on the ion chromatograms from six control samples. The HPLC-MS/MS method used to determine the benzylpenicillin in bovine milk and plasma was validated during the assay. Linearity was verified from 2 ng/mL to 400 ng/mL of benzylpenicillin in plasma. The extraction recovery from 6 spiked plasma samples showed that benzylpenicillin and phenoxymethylpenicillin were efficiently extracted from bovine plasma (mean recovery 69.8%, coefficient of variation (CV) 3.9%). The matrix effect after replicate analyses (n=2) on benzylpenicillin and phenoxymethylpenicillin from plasma was deemed negligible (-6.3% and +2.7% respectively). The precision and accuracy evaluated at 3 concentration levels of benzylpenicillin were within an acceptance range. The highest

acceptable CV was set at 20% for the quality control (QC) LLOQ and 15% for other concentrations. The highest acceptable percentage of error was set at +/- 20% for the LLOQ and +/- 15% for other concentrations. Milk QC samples would be rejected if the concentration deviated from the theoretical value by more than -30% to +10%, or if an unsatisfactory chromatographic peak was obtained, or in the event of a manipulation error. During each batch analysis, at least 4 of the 6 QC samples were within this range, and at least 5 calibration points were included in the final calculation of the calibration curve parameters. Analysis of milk and plasma specimens was deemed acceptable as the calibration curve and the quality controls included in the analytical batches were within their respective acceptance criteria.

Data analysis

Statgraphics Plus® (Statistical Graphics, Rockville, USA) was used for statistical analysis for calibration. The linearity of the response in plasma and milk was evaluated with 3 calibration curves. Each calibration curve included a blank matrix, a zero matrix and six levels of concentration (2, 5, 25, 75, 200 and 400 ng of benzylpenicillin per mL of plasma). The calibration curve was obtained by least-squares regression of the calculated response versus theoretical concentration using several regression models (untransformed, $1/x$, $1/x^2$). ANOVA, including the lack-of-fit test, was performed on the regression curve, and linearity was determined based on examination of the residuals; outliers were checked and discarded when the absolute value of the residual was above 2. Linear adjustment by the least-squares mean method with a weighting factor of $1/x^2$ was determined to be the most appropriate model to express the relationship between the response and the theoretical concentration in bovine plasma. One outlier was discarded.

Areas Under the Curve (AUC) were calculated for benzylpenicillin concentrations in blood and milk using Microsoft Excel®. Concentrations in blood and milk were summarized at each time point into means and geometric means; the latter to reduce the influence of possible outliers. Benzylpenicillin concentrations in milk were log-transformed for easier visual interpretation. Further statistical analysis was performed using SAS® software version 9.4 (SAS Institute Inc., Cary, NC, USA). Results were considered significant at alpha=0.05 level. A simple linear regression model was fitted with AUC as the outcome variable and "actual administered dose" as an explanatory variable. A non-parametric test (the Jonckheere-Terpstra test; (Terpstra & Magel, 2003) was also performed at the given time points to evaluate whether benzylpenicillin concentrations in plasma and milk differed between treatment groups. Measurements that were below the quantification limit were treated as zero in the analysis.

Results

No adverse reactions, clinical signs or changes in daily milk production were observed after treatment. The mean dose of penicillin actually administered to each treatment group is shown in Table 1.

Concentration in plasma

The mean benzylpenicillin concentrations determined in the plasma over 24 hours are presented in Figure 1. A linear dose-dependent increase was seen in the mean AUCs for benzylpenicillin in plasma (corrected by the actual administered dose in IU/kg bodyweight; $p<0.0001$) (Figure 2). The Jonckheere-Terpstra test demonstrated that increasing the dose increased the benzylpenicillin in the plasma at 6, 12, 15 and 18 hours after dosing (p-values 0.0002; <0.0001; 0.0001; and 0.0037 respectively). The concentration in the plasma dropped below the quantification limit at 24 hours in all dosage regimens (data not shown).

Include Figure 1 and Figure 2 here

Concentration in milk

The mean and geometric mean of benzylpenicillin concentrations in samples of milk from treated quarters are presented in Table 2. Mean log concentrations of benzylpenicillin in milk are presented in Figure 3. At the first milking, 12 hours after dosing, there was evidence that increasing the benzylpenicillin dose administered to the mammary quarter increased milk benzylpenicillin concentrations ($p=0.002$ in the Jonckheere-Terpstra test). The difference was not significant at the second milking, 24 hours after dosing, and fourth milking, 48 hours after dosing ($p=0.234$ and $p=0.500$ respectively). A linear dose-dependent increase was seen in the mean AUCs of benzylpenicillin concentrations in milk (corrected by the actual administered dose in IU/kg bodyweight; $p=0.046$) (Figure 4).

Include Table 2 and Figure 3 here

Discussion and conclusion

The aim of intramammary benzylpenicillin administration is to achieve concentrations of the active ingredient above the MIC for the relevant pathogens. These concentrations should be reached at the site of infection (in the udder) for long enough to eliminate the infection, while minimizing the exposure of commensal bacteria (such as those found in the gut) to the drug to reduce the risk of selecting resistant bacteria . We believe that this is the first validated study on the pharmacokinetics of benzylpenicillin after intramammary administration.

In Europe, the most relevant Gram-positive udder pathogens isolated in mastitis and treated with antimicrobials are *Staphylococcus aureus* and *Streptococcus uberis* (Tenhagen, Hansen et

al., 2009; Verbeke, Piepers et al., 2014; Santman-Berends, Lam et al., 2015; Vakkamäki, Taponen et al., 2017). No clinical MIC breakpoints are available for penicillin in bovine mastitis. The epidemiological breakpoints for the susceptibility of staphylococci (including *S. aureus*), group A streptococci (*S. dysgalactiae*) and group B streptococci *(S. agalactiae)* to penicillin are defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) as 0.125 µg/mL. No such breakpoint is available for *S. uberis* (EUCAST, 2017), although 0.125 µg/mL is commonly accepted (Thomas, de Jong et al., 2015).

Most mastitis-causing streptococci have remained susceptible to benzylpenicillin (Hendriksen, Mevius et al., 2008; MARAN, 2008; SVARM, 2010; FINRES, 2015). Reduced susceptibility has been reported in a small proportion of *S. uberis* isolates (Haenni, Galofaro et al., 2010; FINRES, 2015). This finding probably has no clinical relevance, because the proportion of these isolates remains below 5% and their MIC is still much below the concentrations achieved by intramammary administration of penicillin (FINRES, 2015). *S. aureus* has developed penicillin resistance by producing β-lactamase. The proportion of β-lactamase-positive isolates collected from quarter milk samples of cows with mastitis in European countries varies from less than 10% to over 50% (Hendriksen, Mevius et al., 2008; Kalmus, Aasmäe et al., 2011; FINRES, 2015; Thomas, de Jong et al., 2015). Clinical mastitis caused by coagulase-negative staphylococci can also be treated with penicillin, and resistance among these is more common than in *S. aureus* (Taponen, Nykäsenoja et al., 2016). It is remarkable that Scandinavian countries which almost exclusively treat mastitis with benzylpenicillin have reported consistently very low or decreasing numbers of penicillin-resistant *S. aureus* isolates (Pitkälä, Haveri et al., 2004; Swedres-SVARM, 2014; FINRES, 2015; NORM-VET, 2016).

238 In the current study, penicillin concentration in the milk compartment exceeded the MIC for
239 the relevant pathogens for at least 24 hours with all dosages. 24 hours is proposed to be a
240 good administration interval for an intramammary antibiotic, since it requires only once a day
241 manipulation of the teat and ensures compliance due to adaptation to different milking
242 regimes (twice-a-day, three-times-a-day or voluntary milking systems). With the lower dose
243 of 200 000 IU, the concentration at 24 hours was as much as 20 times greater than the MIC;
244 with 300 000 IU, 22 times greater; and with 600 000 IU, 70 times greater. Variation in the
245 600 000 IU group was large, so the geometric mean, which was 35 times greater than the
246 MIC, may describe the results more accurately.

247 For time-dependent antibiotics such as benzylpenicillin, maximum kill *in vitro* is achieved at
248 3 to 4 times the MIC, and the most important predictor for elimination of infection is the time
249 above the MIC (Turnidge, 1998). In light of the paradoxically reduced bactericidal activity of
250 high doses of penicillin (first described by Eagle and Musselman (1948), and known as the
251 “Eagle effect”), our study indicates that all doses used could possibly be unnecessarily high.
252 It must be noted that the paradoxically inferior effect of high doses has been observed with
253 most β-lactam antibiotics, mainly with Gram-positive bacteria, and it is not always
254 synonymous with the classic Eagle effect (Odenholt-Tornqvist, 1988; Hamilton-Miller &
255 Shah, 1999). Penicillin has been used to treat mastitis for over 50 years, at lower doses than
256 at present (Le Louedec, 1978) and early studies showed that doses as low as 100 000 IU
257 (every milking) resulted in high cure rates (Edwards, 1962; Le Louedec, 1978). However,
258 the main target pathogen at the time of those studies was *S. agalactiae,* which responded
259 very well to treatment (Edwards, 1962).

260 There are more arguments to support the higher dosing regimen. Some caution is warranted
261 when interpreting the results, as the only samples taken consisted of foremilk. Studies with
262 another β-lactam antibiotic (cephapirin) have shown that foremilk contains higher

concentrations of the drug than subsequent milk, potentially overestimating the drug concentration reached in the entire udder (Stockler, Morin et al., 2009).

The results of the current study also reveal considerable variation in benzylpenicillin concentrations in milk between cows in the same treatment group. The same finding was reported by Bjorland, Waage et al. (1998) who compared two intramammary doses of benzylpenicillin in five cows. Moreover, one target pathogen, *S. aureus,* is known to penetrate into udder tissue (Almeida, Matthews et al., 1996), and penicillin is also transferred into this tissue in the usual pharmacokinetic manner (Ziv, 1980b). Penicillin concentrations in udder tissue were not measured in this study, so we can only speculate on the penetration of penicillin into deeper udder tissue and whether effective concentrations accumulate there. However, Ehringer and Kietzmann (2000) noticed that the concentration of benzylpenicillin in the tissue decreased exponentially with increasing vertical distance from the teat base, and they found that benzylpenicillin concentration in the tissues of the lower part of the udder was around $1/10^{th}$ of that of the upper part of the udder. This could be the reason why a field trial which studied clinical mastitis caused by *S. aureus* demonstrated significantly better cure rates when intramammary treatment was supplemented with systemic penicillin, compared to intramammary treatment alone (Taponen, Jantunen et al., 2003).

Finally, the pharmacokinetics of benzylpenicillin in healthy animals could be significantly different from cows suffering from mastitis, as the pharmacokinetic properties of active substances are determined in healthy animals (EMA, 2000). Pathological changes in cows with clinical mastitis, including swelling and occlusion of the ducts in the mammary gland, may result in uneven distribution of the drug and the associated risk of lower local penicillin concentrations (Ullberg, Hansson et al., 1958; Ziv, 1980b). In an experimental *S. uberis* infection, inflammatory changes caused widespread occlusion of the mammary gland secretory system (Pedersen, Aalbaek et al., 2003). Inflammation in the udder changes the

composition of the milk and damages the blood-milk barrier, increasing the permeability of the blood and milk compartments and potentially accelerating elimination of the drug (Erskine, Wilson et al., 1995; Zhao & Lacasse, 2008).

In conclusion, our results showed a dose-dependent increase in the concentration of benzylpenicillin in plasma and milk when applied in an oily suspension with micronized particles. Although concentrations in the milk after administration of the lower doses were well above the MIC for the target pathogens, distribution of the active ingredient in the inflamed udder and the location of the pathogens inside the udder tissue must be taken into account. For this reason, the suggested dose for this product is the higher dose of 600 000 IU administered once a day. It would be useful to conduct field studies with naturally occurring intramammary infections to define the optimum dose and duration of intramammary treatment with benzylpenicillin.

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Authors' Contribution Statement

EA summarized the results, analyzed the data and drafted the text of the manuscript. SP and PRJ drafted the text and provided expertise particularly in pharmacokinetical, pharmacodynamical and statistical aspects. VM designed and monitored the study and reviewed the text.

Conflict of Interest

Satu Pyörälä and Päivi Rajala-Schultz have no affiliations with or involvement in any organization or entity with any financial interest. Elke Abbeloos and Vesa Myllys work for companies that market and/or distribute the product described in this article.

Tables and figures

Table 1: Mean (and SD) body weight (kg), age (months), days in milk (days) and daily milk yield (kg) for each of the treatment groups and the theoretical dose of benzylpenicillin. The last column contains the actual dose of benzylpenicillin given after weighing of the intramammary tubes after administration

Treatment group	Theoretical dose of benzylpenicillin (IU)	Cow body weight (kg)		Age (months)		Days in Milk		Daily milk yield (kg)		Actual dose of benzylpenicillin (IU)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	200 000	585.4	55.5	40.8	12.3	149.2	34.2	32.3	7.9	189 832.0	4613.0
2	300 000	622.8	74.2	46.4	14.0	156.2	23.3	32.4	5.1	289 994.0	8404.0
3	600 000	615.2	43.1	42.8	18.3	151.0	36.7	32.8	7.4	610 244.0	5829.0
Average		**607.8**	**57.1**	**43.3**	**14.2**	**152.1**	**29.7**	**32.5**	**6.4**		

Table 2: Mean, standard deviation and geometric mean of the concentration of benzylpenicillin (in ng/mL) found in milk after intramammary administration of approximately 200.000 IU, 300.000 IU or 600.000 IU of benzylpenicillin in the form of an oily suspension with micronised particle size.

Time (h)	Group 1 (200.000 IU)			Group 2 (300.000 IU)			Group 3 (600.000 IU)		
	Mean	SD	GeoMean	Mean	SD	GeoMean	Mean	SD	GeoMean
	(ng/mL)			(ng/mL)			(ng/mL)		
0	5/5 samples below quantification limit			5/5 samples below quantification limit			5/5 samples below quantification limit		
12	35345.24	22572.37	30438.00	41351.25	16982.59	38558.00	103476.36	45736.28	96380.47
24	2519.24	626.19	2459.06	2843.88	1160.82	2648.71	8778.28	12314.25	4486.51
48	13.86	10.18	11.19	18.77	19.36	11.12	20.09	8.85	18.37
72	5/5 samples below quantification limit			3/5 samples below quantification limit			5/5 samples below quantification limit		

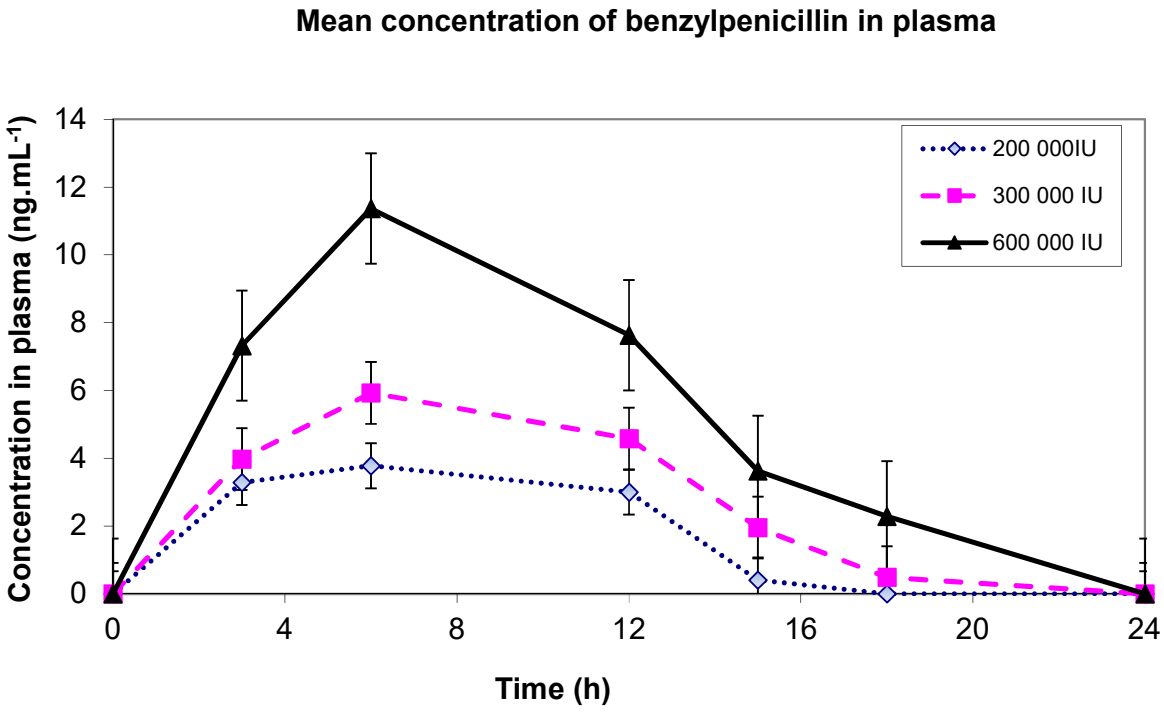
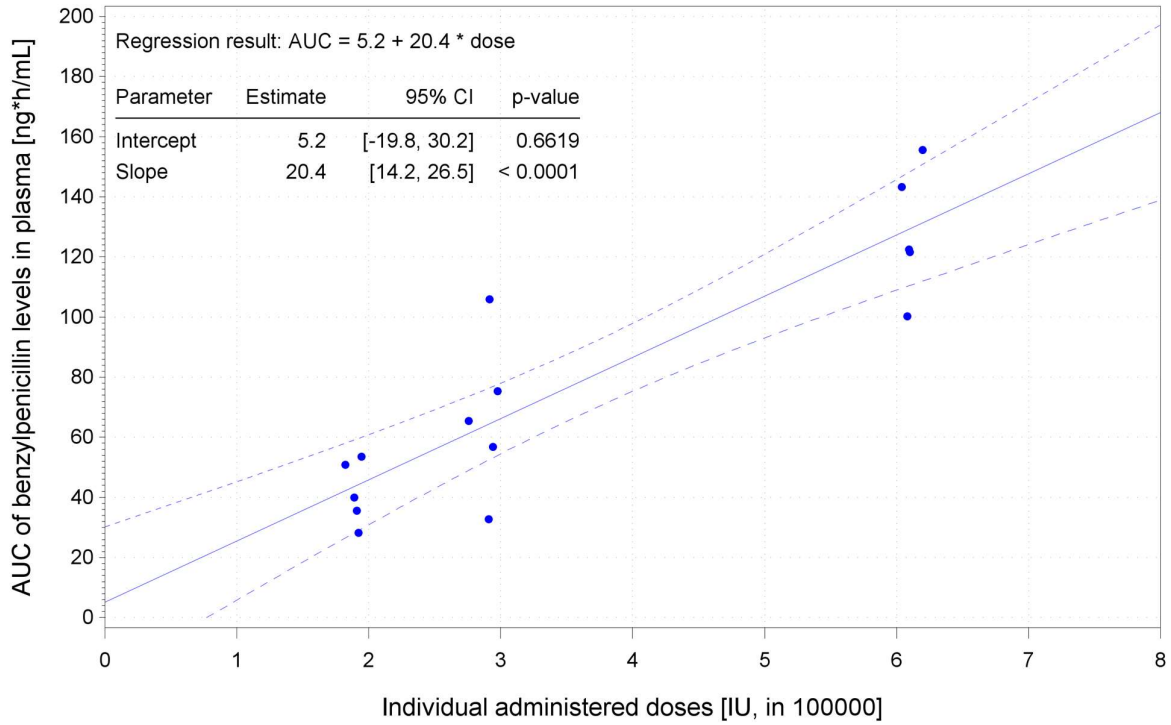


Figure 1: Mean benzylpenicillin concentrations in ng/mL (with SEM) determined in plasma specimens after intramammary administration of approximately 200.000 IU, 300.000 IU or 600.000 IU of procaine benzylpenicillin in the form of an oily suspension with micronised particle size.



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453 **Figure 2**: Simple linear regression model of Mean Area Under Curve (AUC) of
454 benzylpenicillin levels in plasma after intramammary administration of approximately
455 200.000 IU, 300.000 IU or 600.000 IU benzylpenicillin in the form of an oily suspension with
456 micronised particle size. The AUC was corrected for the actual administered dose
457 administered to each cow in IU/kg bodyweight.

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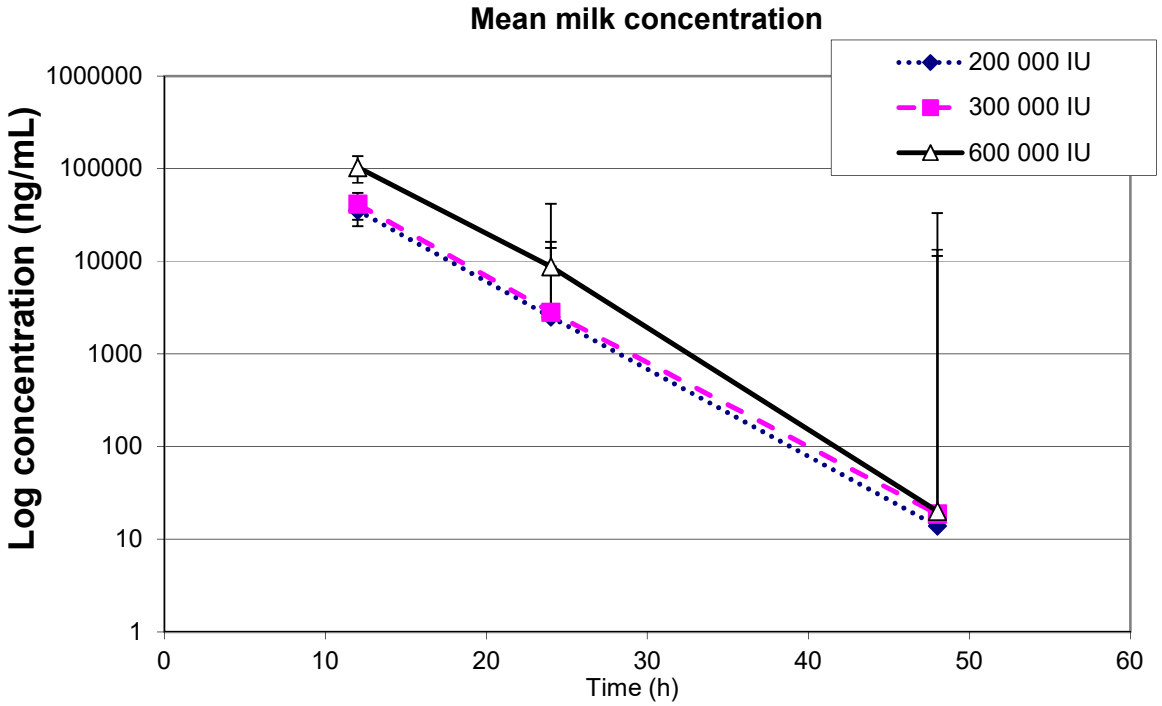

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462 **Figure 3**: Mean benzylpenicillin log concentrations (ng.mL^{-1}) (with SEM) found in quarter
463 milk samples after intramammary administration of one quarter with respectively 200.000
464 IU, 300.000 IU and 600.000 IU of benzylpenicillin in the form of an oily suspension with
465 micronized particle size.

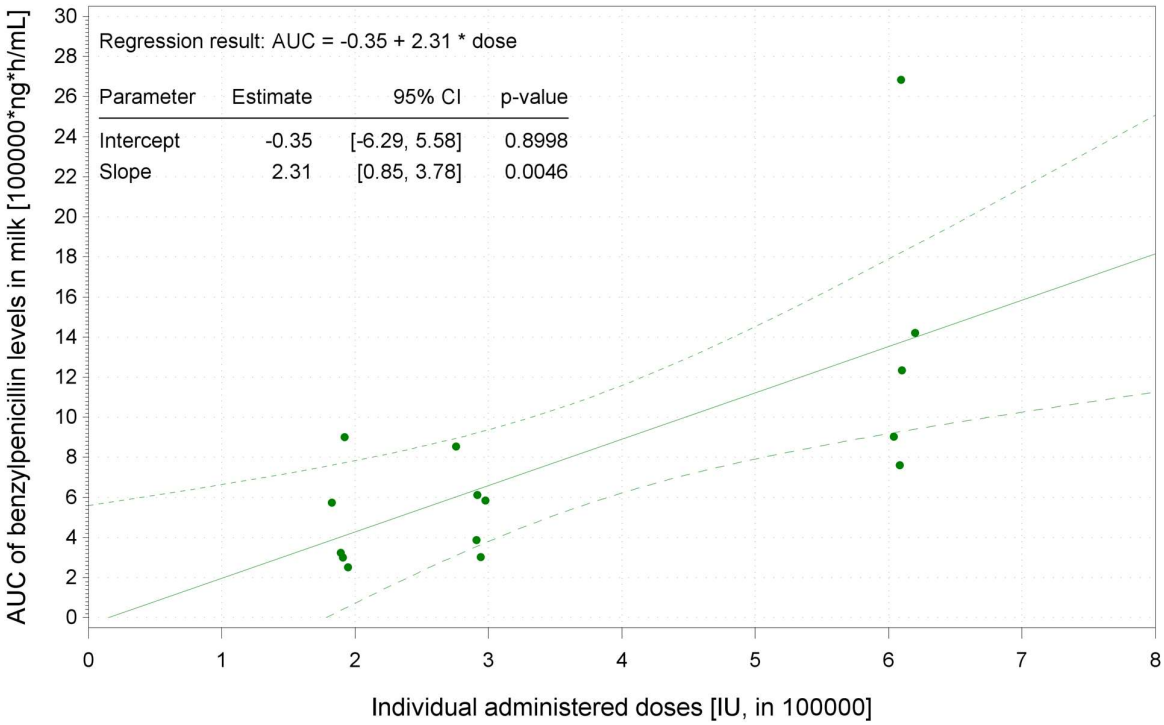

Figure 4: Simple linear regression model of Mean Area Under Curve (AUC) of benzylpenicillin levels in milk after intramammary administration of approximately 200.000 IU, 300.000 IU or 600.000 IU benzylpenicillin in the form of an oily suspension with micronised particle size. The AUC was corrected for the actual administered dose administered to each cow in IU/kg bodyweight.