Tissue Depletion of Amoxicillin and Its Major Metabolites in Pigs: Influence of the Administration Route and the Simultaneous Dosage of Clavulanic Acid

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A residue depletion study of amoxicillin (AMO) and its major metabolites, amoxicilloic acid (AMA) and amoxicillin diketopiperazine-2′,5′-dione, was performed after a single oral (p.o.) and intravenous (i.v.) administration of amoxicillin (20 mg kg^{-1}) and amoxicillin/clavulanic acid (20 and 5 mg kg^{-1}) to pigs. Animals were slaughtered 12, 36, 48, 60, 72, and 84 h after dosing. Tissue samples were analyzed using liquid chromatography-tandem mass spectrometry. Kidney samples contained high concentrations of amoxicilloic acid metabolite, which depleted much slower from tissues than amoxicillin, both after p.o. ($t_{1/2AMO} = 4.5$ h vs $t_{1/2AMA} = 8$ h) and i.v. ($t_{1/2AMO} = 4$ h vs $t_{1/2AMA} = 8$ h) administration. Moreover, after oral administration, significantly higher amoxicilloic acid concentrations were measured in liver and kidney than after i.v. administration. The coadministration of amoxicillin with clavulanic acid provoked no significant differences in amoxicilloic acid tissue concentrations as compared to an amoxicillin dosing. The prolonged presence of residues of amoxicilloic acid in edible tissues can play an important role in food safety, because the compound could give rise to a possible health risk, although it is not included in the maximum residue limit legislation.

KEYWORDS: Amoxicillin; amoxicilloic acid metabolite; residue depletion; risk assessment

INTRODUCTION

Amoxicillin is a β-lactam antibiotic with a broad activity against Gram-negative and Gram-positive bacteria and with a fairly good penetration into tissues (*1*). It has a high activity against pathogens in pigs, such as *Actinobacillus pleuropneumoniae*, *Streptococcus suis*, *Pasteurella multicoda*, and *Escherichia coli* (*2*). Among the various classes of antibiotics, penicillins are the most frequently used agents in the treatment of bacterial infections. The intense use of amoxicillin in veterinary practice can give rise to an increasing risk for human and animal health. Therefore, the European Union (EU) has established a maximum residue limit (MRL) of 50 μg kg^{-1} for amoxicillin in porcine edible tissues (kidney, liver, muscle, and fat) (*3*). The marker residue for the MRL is the parent compound amoxicillin, as is also the case for other penicillins. However, there is another possible health risk when dealing with penicillins.

Penicillins are known to cause most allergic drug reactions; it is estimated that the overall prevalence of allergy to penicillin in different human populations is 3–10% (*4*). There is a risk that residues of hypersensitivity-inducing drugs, such as penicillins, may elicit allergic reactions in human consumers of food of animal origin (*4*). Allergic reactions to foods containing residues of penicillin are generally restricted to dermatological reactions such as urticaria, skin rashes, polymorphic exanthems, asthma, and hypotension (*5*). However, there are reports of life-threatening anaphylactic shock reactions in (pre)sensitized subjects after consumption of food (meat and milk) containing penicillin residues (*4–10*).

The mechanisms of allergy to penicillins are similar to the normal humoral antibody response to exogenous macromolecules. To become haptens, drugs that stimulate an immunologic hypersensitivity response must combine with a carrier molecule. During metabolization, the β-lactam ring of penicillins is opened, resulting in the formation of a highly reactive molecule (penicilloic acid−amoxicilloic acid in the case of amoxicillin) (*11*). This molecule irreversibly forms an amide linkage with adjacent proteins to form a penicillol hapten (major determinant). The haptens derived from penicillin are shown in **Figure 1** (*12*). The penicilloic acid metabolites can be formed by enzymatic hydrolysis with β-lactamase and in acidic or alkaline media (*13, 14*). These β-lactamase enzymes are produced by resistant bacteria (*14*) or by the normal healthy intestinal microflora (*15*). The degradation of amoxicillin into its amoxicilloic acid metabolite can also be caused by the action of acidic intestinal juices and enzymes (*16*).

For antibiotics, a well-suited residue monitoring system for meat and meat products consists of at least four stages (*17*): a prescreening test (bacterial inhibition tests), a selective screening test (immunoassays or receptor assays), a chemical identification

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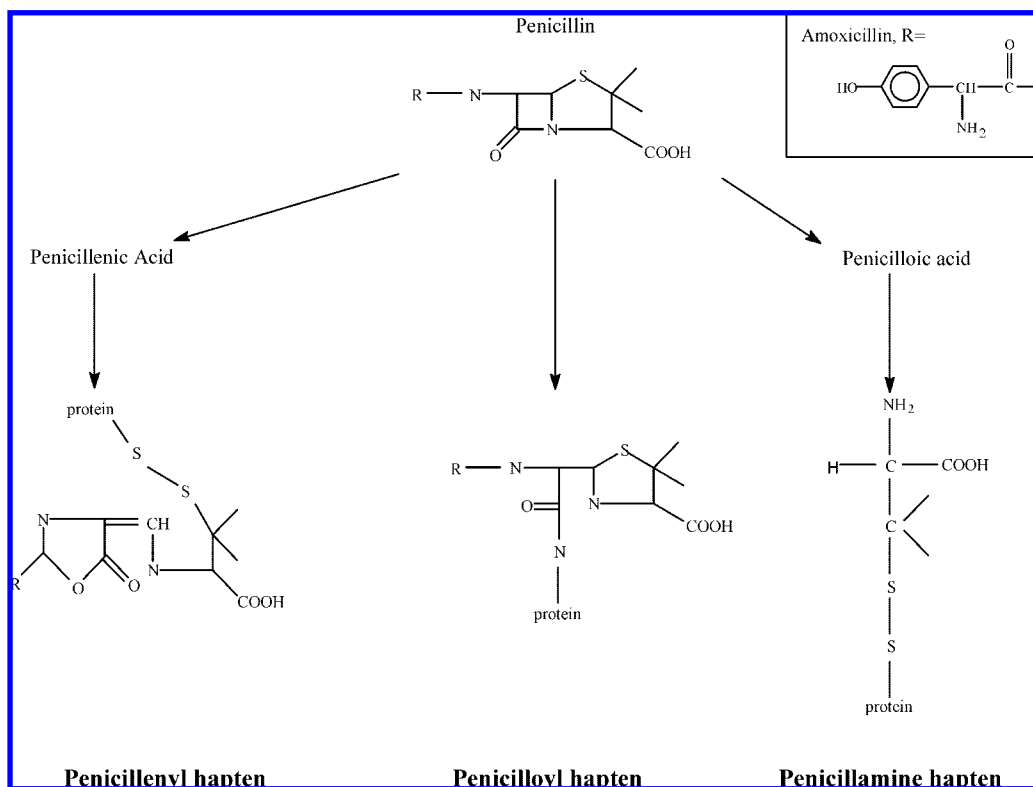
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Figure 1. Haptens derived from penicillin (Chowdhury and Lieberman, 1999; *12*).

with liquid chromatography or gas chromatography with mass spectrometric detection, and finally, a quantitative assay of the identified residues in view of the established MRL (*3*). These current bacterial inhibition tests only measure the intact penicillin structure and fail to have the ability to detect substances that lack antibacterial effect but could have allergic properties (*18*). As a result, Huber (*18*) mentioned that an underestimation of the real penicillin concentration in the samples could occur. Also, the chromatographic methods with ultraviolet (*19, 20*), fluorimetric (*21*), and mass spectrometric (*22–25*) detection of β-lactams in animal tissues only measure the parent compounds and not the penicilloic acid metabolites. De Baere et al. developed a method for the quantitative analysis of amoxicillin, amoxicilloic acid, and amoxicillin diketopiperazine-2′,5′-dione in animal tissues (*26*). They described a prolonged presence of the amoxicilloic acid metabolite in kidney and liver samples from pigs that received amoxicillin via drinking water, but no withdrawal times were calculated.

Amoxicilloic acid and amoxicillin diketopiperazine-2′5′-dione are the two major metabolites of amoxicillin (*13, 27*). Nägele and Moritz mentioned amoxicillin diketopiperazine-2′5′-dione as a possible further degradation product of amoxicilloic acid metabolite (*13*). There is no mention of an amoxicillin conjugate metabolite in the literature, so a specific hydrolysis of tissue samples for a subsequent residue study is not necessary. Indeed, none of the above-mentioned bioanalytical methods include acid or alkaline hydrolysis or enzymatic digestion (*19–25*).

The objective of the present study was to characterize and compare the tissue depletion of amoxicillin and its major metabolites in edible tissues after oral and intravenous administration of amoxicillin and amoxicillin/clavulanic acid to pigs. To our knowledge, this is the first time that a full residue depletion study was performed for the major metabolites of amoxicillin in edible porcine tissues.

MATERIALS AND METHODS

Animals and Experimental Design. The residue study was approved by the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University and was conducted with 56 healthy Belgian Landrace stress-negative pigs, weighing 35.6 ± 10.2 kg at the start of the experiment. The pigs were housed in 14 groups of 4 pigs that had access to water *ad libitum* and were fed with antibiotic-free commercial feed (Versele Laga, Deinze, Belgium). A 7-day acclimatization period was applied prior to the study and the pigs were fasted for at least 12 h before administration of the drugs.

At day 0, eight pigs were intravenously (i.v.) treated with a combination of amoxicillin/clavulanic acid at a dosage of 25 mg kg^{-1} body weight (20 mg kg^{-1} of amoxicillin and 5 mg kg^{-1} of clavulanic acid, Augmentin Soluble Powder, GlaxoSmithKline, Worthing, United Kingdom) and slaughtered at 12 ($n = 4$) and 36 h ($n = 4$) after dosing. Twenty pigs received an i.v. administration of amoxicillin alone at a dosage of 20 mg kg^{-1} body weight (Clamoxyl Soluble Powder, GlaxoSmithKline) at day 0. These pigs were sacrificed at 12 ($n = 4$), 48 ($n = 4$), 60 ($n = 4$), 72 ($n = 4$), and 84 h ($n = 4$) after drug administration. The i.v. bolus was given through a catheter in an ear vein.

For the oral (p.o.) study, eight pigs received a combination of amoxicillin/clavulanic acid at a dosage of 25 mg kg^{-1} body weight (20 mg kg^{-1} of amoxicillin and 5 mg kg^{-1} clavulanic acid, Augmentin oral tablets, GlaxoSmithKline) at day 0 and were slaughtered at 12 ($n = 4$) and 36 h ($n = 4$) after dosing. Twenty pigs received an oral dose of amoxicillin alone at 20 mg kg^{-1} body weight (Amoxycilline 70%, KELA, Hoogstraten, Belgium) at day 0. These pigs were sacrificed at 12 ($n = 4$), 48 ($n = 4$), 60 ($n = 4$), 72 ($n = 4$) and 84 h ($n = 4$) after drug administration. The oral bolus was administered through a stomach tube after dissolving the powder/tablet in tap water.

After slaughter, the two whole kidneys and about 100 g of liver, fat, and muscle were collected separately, to avoid contamination, minced, homogenized using a Robot-coupe mixer (Robot-coupe, Mont-Ste-Geneviève, Belgium) and frozen in plastic bags at ≤ -70 °C, pending analysis. All samples were analyzed within three months after sampling.

Table 1. Mean Tissue Concentrations of Amoxicillin (AMO), Amoxicilloic Acid (AMA), and Amoxicillin Diketopiperazine-2′,5′-dione (DIKETO) in Pig Kidney, Liver, Fat, and Muscle[a]

h	route	kidney (ng g^{-1})			liver (ng g^{-1})			fat (ng g^{-1})			muscle (ng g^{-1})		
		AMO	AMA	DIKETO	AMO	AMA	DIKETO	AMO	AMA	DIKETO	AMO	AMA	DIKETO
12	p.o.	618 (359)	10132[b] (3096)	88 (61)	<LOQ	1379[c] (201)	<LOQ	<LOQ	127 (68)	<LOD	<LOQ	30 (17)	<LOQ
	i.v.	915 (148)	5575[b] (744)	47 (23)	<LOQ	546[c] (198)	<LOQ	39 (20)	118 (66)	<LOD	35 (18)	32 (22)	<LOQ
48	p.o.	<LOD	205 (115)	<LOD	<LOD	35 (14)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	i.v.	<LOD	100 (79)	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
60	p.o.	<LOD	231 (115)	<LOD	<LOD	42 (24)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	i.v.	<LOD	120 (40)	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
72		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
84		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

[a] Measured at 12 ($n = 4$), 48 ($n = 4$), 60 ($n = 4$), 72 ($n = 4$), and 84 h ($n = 4$) after Bolus Administration of 20 mg kg^{-1} Amoxicillin (oral and intravenous administration). LOD = 1.7, 7.1, and 2.7 ng g^{-1} for AMO, AMA, and DIKETO, respectively, in porcine kidney; 3.5, 14.2, and 1.6 ng g^{-1} for AMO, AMA, and DIKETO, respectively, in porcine liver; 1.5, 11.1, and 0.9 ng g^{-1} for AMO, AMA, and DIKETO, respecitvely, in porcine muscle, and 1.7, 10.6, and 0.8 for AMO, AMA, and DIKETO, respectively, in porcine fat. LOQ = at least 25 ng g^{-1} for all components in all tissue matrices. [b] Significant $p = 0.025$. [c] Significant $p = 0.001$.

Analytical Method and Validation. *Chemicals and Reference Substances.* Amoxicillin trihydrate and ampicillin were chemical reference substances (CRS) of the European Pharmacopoeia (Council of Europe, Strasbourg, France). The standards of amoxicilloic acid sodium salt and amoxicillin diketopiperazine-2′,5′-dione were obtained from LGC Promochem SARL (Molsheim, France). Separate stock solutions at 1 mg mL^{-1} were prepared in HPLC water for amoxicillin, amoxicilloic acid, and ampicillin, respectively, and in a mixture of HPLC water and methanol (50/50, v/v) for amoxicillin diketopiperazine-2′,5′-dione. All stock solutions were stored at ≤ −70 °C and found to be stable for at least six months. The stock solutions were divided into small portions of about 200 μL each in amber colored Eppendorf cups (Novolab, Geraardsbergen, Belgium). On each analysis day, a new cup was thawed and discarded after use. Acetonitrile and water were obtained from Acros (Geel, Belgium) and were of HPLC grade. Formic acid and potassium dihydrogen phosphate (KH$_2$PO$_4$), used for extraction, were of analytical grade. Amicon Microcon YM-30 Centrifugal Devices (molecular weight (MW) cutoff: 30 kDa) and IC Millex-LG 0.20 μm filters were both from Millipore (Bedford, Massachusetts, USA).

LC-MS/MS System. The HPLC system consisted of an Alliance type 2695 separations and a column heater, both from Waters (Millford, Massachusetts, USA). The temperature of the autosampler was set at 5 °C, and the column was kept at room temperature. Chromatographic separation of the analytes was achieved using a PLRP-S polymeric column (150 mm × 2.1 mm i.d., 100 Å, 3 μm) protected by a guard column of the same type (5 mm × 3.0 mm i.d.), both from Polymer Laboratories (Shropshire, UK). The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). A gradient elution was performed at a flow-rate of 0.2 mL min^{-1}, that is, 0–1.9 min, 2% B; 2–5 min, 20% B; 5.1–12 min, 50% B; 12.1–20 min; 2% B. A Quattro Ultima triple quadrupole instrument (Millford) was used in the positive ionization MS/MS mode. The following tune parameters were used for amoxicillin, amoxicilloic acid, amoxicillin diketopiperazine-2′,5′-dione, and ampicillin: capillary, 3.50 kV; cone, 25 V; source temperature, 120 °C; desolvation temperature, 250 °C; cone gas flow, ±50 L h^{-1}; desolvation gas flow, 750 L h^{-1}; resolution (LM1, HM1, LM2, and HM2), 15.0; ion energy 1, 1.0; ion energy 2, 3.0; entrance, −1; exit, 1; multiplier, 650 V; collision gas, argon (Pirani pressure, ±3.4 × 10^{-3} mbar); and dwell time, 0.2s. The optimal settings for collision energy, corresponding to a (nearly) 100% fragmentation of the precursor ions, were optimized for each component. Quantification was performed with the MassLynx software using the following transition for amoxicillin: 365.9 > 348.8; amoxicilloic acid: 383.9 > 322.8; diketopiperazine-2′,5′-dione: 365.9 > 159.9, and ampicillin: 349.9 > 159.9.

The method was fully validated according to EU requirements for amoxicillin, amoxicilloic acid, and diketopiperazine-2′,5′-dione (linearity, precision, trueness, quantification limit (LOQ), detection limit (LOD), and specificity) (28). The LOQ was at least 25 ng g^{-1} for all components in the different tissue matrices. This is at least half of the MRL of amoxicillin (25 ng g^{-1}), as required by the EU guideline (28). The LOD was determined as the lowest measured content from which

it is possible to deduce the presence of the analyte with reasonable statistical certainly, using the criterion of the signal-to-noise (S/N) ratio of 3/1. Special attention was paid to the stability of the components during extraction and storage, including short-term stability, long-term stability, stock solution stability, and postpreparative stability (29).

Tissue Extraction. The extraction of amoxicillin, amoxicilloic acid, and amoxicillin diketopiperazine-2′,5′-dione from the tissue samples was performed according to two reports with some modifications (24, 26). Briefly, 1 g of tissue sample was weighed into a 50 mL polypropylene centrifuge tube and was spiked with the internal standard ampicillin (25 μL of a working solution of 10 μg mL^{-1}). Thereafter, 7 mL of a 10 mM KH$_2$PO$_4$ (pH 4.5) solution were added, and the sample was mixed well for 30 s using a vortex mixer. The sample was extracted for 20 min on a rotary mixer, followed by a 10 min centrifugation step (4500 rpm) at 4 °C. Then, 1.5 mL of the extract were transferred to an Eppendorf tube (Novolab) and centrifuged for 10 min at 13 000 rpm at 4 °C. A 500 μL aliquot of the supernatant was used for the ultrafiltration step using a Microcon YM-30 Centrifugal Filter Device (Millipore, Bedford, Massachussetts, USA). After centrifugation for 30 min at 13 000 rpm at 4 °C, the filtrate was poured through a 0.20 μm Millex filter (Millipore) into an autosampler vial, and a 10 μL aliquot was injected onto the LC column.

Statistical Analysis. Means and standard deviations (SD) were calculated for all data points. Student's t-test was used to compare the tissue concentrations after both p.o. and i.v. administration of amoxicillin and amoxicillin/clavulanic acid ($\alpha = 0.05$). The withdrawal time was estimated by linear regression analysis of log-transformed tissue concentrations and was determined at the time when the 95% upper one-sided tolerance limit was below the MRL with 95% confidence (29).

RESULTS AND DISCUSSION

Residue Depletion. The mean concentrations (ng g^{-1}, ±SD) of amoxicillin, amoxicilloic acid, and amoxicillin diketopiperazine-2′,5′-dione in kidney, liver, fat, and muscle after oral and intravenous administration of 20 mg kg^{-1} amoxicillin and of 20 mg kg^{-1} amoxicillin/5 mg kg^{-1} clavulanic acid at 12, 36, 48, 60, 72, and 84 h after cessation of medication are presented in **Tables 1** and **2**, respectively.

Twelve hours after both p.o. and i.v. administration, amoxicillin concentrations in kidney samples were relatively high, but decreased rapidly, and 36–48 h after the administration, amoxicillin concentrations were below the quantification limit of 25 ng g^{-1} in all tissue samples. It is remarkable that the amoxicilloic acid metabolite remains much longer in kidney tissue. Sixty hours after cessation of medication, mean amoxicilloic acid concentration was 231 ng g^{-1} after p.o. treatment and 120 ng g^{-1} after i.v. administration. Moreover, in liver tissue the same phenomenon was observed, but residues of amoxicil-

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Table 2. Mean Tissue Concentrations of Amoxicillin (AMO), Amoxicilloic Acid (AMA), and Amoxicillin Diketopiperazine-2′,5′-dione (DIKETO) in Pig Kidney, Liver, Fat, and Muscle[a]

		kidney (ng g^{-1})			liver (ng g^{-1})			fat (ng g^{-1})			muscle (ng g^{-1})		
h	route	AMO	AMA	DIKETO	AMO	AMA	DIKETO	AMO	AMA	DIKETO	AMO	AMA	DIKETO
12	p.o.	477 (171)	10706[b] (2628)	85 (61)	<LOQ	1231[d] (246)	<LOQ	<LOQ	118 (78)	<LOD	39 (38)	41 (4)	<LOQ
	i.v.	429 (421)	3990[b] (2380)	47 (23)	<LOQ	378[d] (260)	<LOQ	<LOQ	231 (47)	<LOD	38 (22)	26 (15)	<LOQ
36	p.o.	<LOQ	1740[c] (785)	<LOQ	<LOQ	239[e] (105)	<LOQ	<LOQ	73 (14)	<LOD	<LOD	<LOQ	<LOD
	i.v.	<LOQ	247[c] (73)	<LOQ	<LOQ	32[e] (13)	<LOQ	<LOQ	25 (19)	<LOD	<LOD	<LOQ	<LOD

[a] Measured at 12 ($n = 4$) and 36 ($n = 4$) hours after bolus administration of 20 mg kg^{-1} amoxicillin and 5 mg kg^{-1} clavulanic acid (oral and intravenous administration). LOD = 1.7, 7.1 and 2.7 ng g^{-1} for AMO, AMA and DIKETO, respectively, in porcine kidney; 3.5, 14.2 and 1.6 ng g^{-1} for AMO, AMA and DIKETO, respectively, in porcine liver; 1.5, 11.1 and 0.9 ng g^{-1} for AMO, AMA and DIKETO, respectively, in porcine muscle and 1.7, 10.6 and 0.8 for AMO, AMA and DIKETO, respectively, in porcine fat. LOQ = at least 25 ng g^{-1} for all components in all tissue matrices. [b] Significant $p = 0.009$. [c] Significant $p = 0.032$. [d] Significant $p = 0.003$. [e] significant $p = 0.03$.
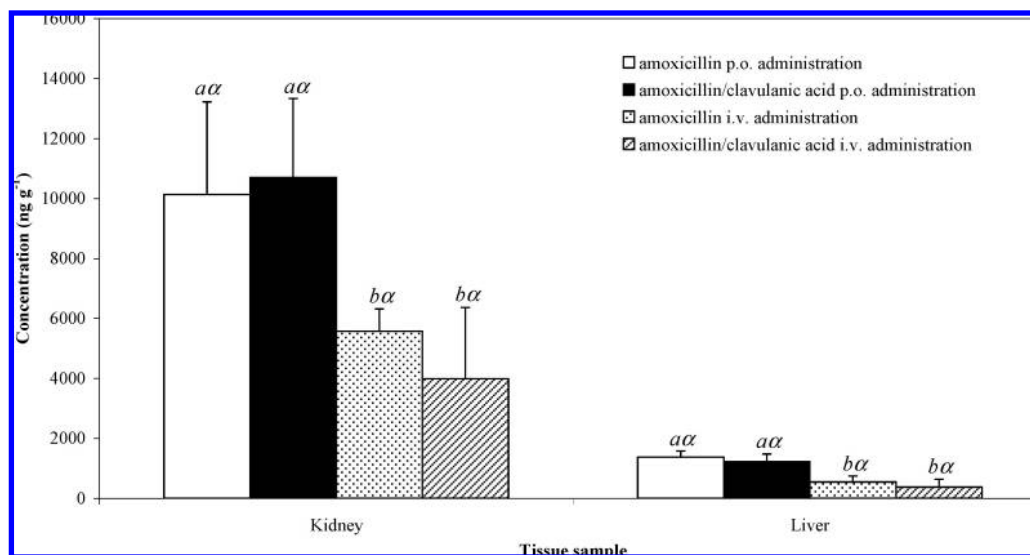
Figure 2. Amoxicilloic acid concentration (mean, +SD) in porcine kidney and liver samples, 12 h after oral (p.o.) administration and intravenous (i.v.) administration of amoxicillin (20 mg kg^{-1}) and amoxicillin/clavulanic acid (20 and 5 mg kg^{-1}) to pigs. Letters *a* and *b* refer to significant differences between administration routes within one treatment ($p < 0.025$). α refers to nonsignificant differences between treatments within one administration route.

loic acid depleted faster than in kidney tissue. In muscle and fat on the other hand, all concentrations of amoxicillin and amoxicilloic acid were below the detection limit at 48 h. Amoxicillin diketopiperazine-2′,5′-dione concentrations higher than the LOQ of at least 25 ng g^{-1} were only determined in kidney samples at the earliest slaughter time of 12 h. In the literature, only a few depletion studies of amoxicillin are reported (*1, 31*), but there is no mention of amoxicilloic acid. Only one publication quotes some preliminary results of the presence of amoxicilloic acid in porcine liver and kidney samples (*26*). The phenomenon of the prolonged presence of amoxicilloic acid in the present study leads to a question regarding risk assessment. It is known that allergic reactions of penicillins in human are of concern in relation to its metabolites (*11*). All existing residue studies include the quantification of the parent penicillin molecule but not its penicilloic acid metabolites. Penicilloic acid metabolites are well-known as the major determinant in hypersensitivity reactions, formed between the carboxyl group of the open β-lactam ring and the amino group of a macromolecule (*18*). Huber (*18*) already mentioned the importance of the penicilloyl−protein complex in edible tissues, because the penicilloyl moiety has the strongest antigenic effect. The residues of the penicilloyl−protein complex could be of much greater significance than residues containing free penicillin molecules, when considering public health hazards. But unfortunately, data are not available regarding the prevalence of penicilloyl−protein complex residues in edible tissues

of slaughtered food animals. In relation to the risk of primary sensitization, it is unlikely that residues could contribute to an overall immune response in view of the low residue levels that are likely to occur in comparison with the high levels during therapeutic use. However, the risk in the case of already sensitized individuals may not be underestimated (*11*). Reports are published of presensitized individuals who developed anaphylactic reactions after consumption of food (meat and milk) containing very low levels of penicillin (*4–10*). It has been estimated that 0.6 μg could cause an allergic reaction in a sensitive adult individual (*4*). A case of a severe anaphylactic shock after consuming pork meat containing 18.6−27 ng g^{-1} of penicillin G was repored (*4*). Dayan (*32*) reported a case of a severe urticaria after the consumption of food containing 0.3−0.6 μg g^{-1} of a penicillin-like substance.

Influence of Administration Route and Coadministration of Clavulanic Acid on Metabolization of Amoxicillin. The difference in amoxicilloic acid concentration (ng g^{-1}, +SD) in kidney and liver after p.o. and i.v. administration of amoxicillin and of amoxicillin/clavulanic acid is presented in **Figure 2**. Statistical evaluation was performed between administration routes within one treatment and between treatments within one administration route. For the evaluation of the influence of administration route, significantly ($p < 0.025$) higher residue concentrations of amoxicilloic acid were present 12 and 36 h post treatment in kidney and liver tissues after p.o. administration in comparison with i.v. administration. Twelve hours after
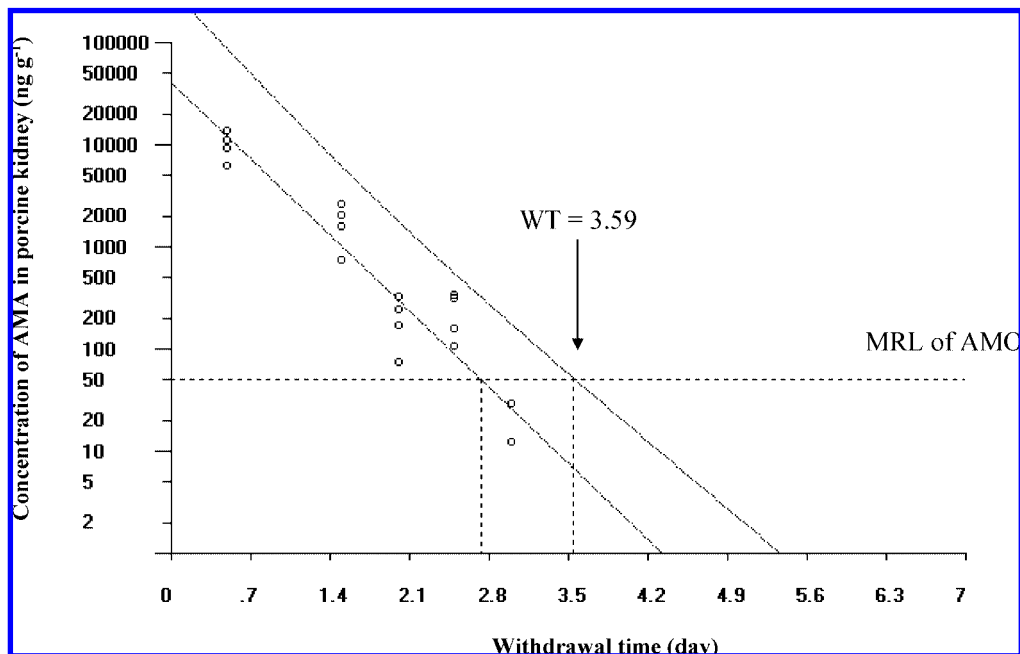
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Figure 3. Plot of the withdrawal time calculation for amoxicilloic acid in swine kidney at the time when the one-sided 95% upper tolerance limit is below the EU MRL for amoxicillin (50 ng g^{-1}) after p.o. administration of amoxicillin.

treatment with an oral dose of amoxicillin alone, the mean residue concentration of amoxicilloic acid was 1379 and 10 132 ng g^{-1} in liver and kidney samples, respectively, and 1231 and 10 706 ng g^{-1} after p.o. administration of amoxicillin/clavulanic acid combination. It is likely from these residue data that substantial degradation of amoxicillin to amoxicilloic acid could take place presystemically after p.o. administration of amoxicillin. Indeed, a previous excretion study in pigs already showed that after p.o. administration of the antibiotic significantly more amoxicilloic acid metabolite could be detected in the urine (*33*). These presystemical effects on amoxicillin would include acid-catalyzed degradation in the stomach and/or enzymatic degradation by intestinal flora and/or intestinal membrane-bound or intracellular enzymes (*31*). Variation in presystemic degradation of amoxicillin could be a possible explanation for the large differences in the oral bioavailability of amoxicillin in pigs, ranging from 10 to 47% (*1, 34–36*), because it is known that amoxicillin is not subjected to a hepatic first-pass effect (*16*).

A publication already mentioned the presence of various β-lactamase enzymes in the normal intestinal microflora of pigs (*15*). Hydrolysis of amoxicillin by β-lactamases results in the formation of amoxicilloic acid (*14*). Clavulanic acid, produced by *Streptomyces clavuligerus*, is a powerful inhibitor of the β-lactamases and is capable of protecting susceptible β-lactam antibiotics from the hydrolytic action of β-lactamases (*14*). From the results in the present study, it can be concluded that inactivation of the β-lactamases by clavulanic acid has no significant influence on the amoxicilloic acid tissue concentration (**Figure 2**). Previous experiments in swine also showed that there were no significant differences in the urinary excretion profiles of amoxicilloic acid after the administration of amoxicillin/clavulanic acid and amoxicillin alone (*33*). Moreover, no significant increase in the oral bioavailability of amoxicillin could be observed after the combined administration of amoxicillin with clavulanic acid to pigs as compared to an amoxicillin dosing alone (*34*).

Withdrawal Time Estimation. The mean amoxicillin concentrations in all matrices were below the LOQ at 36 h after cessation of medication and below the LOD at 48 h after dosing.

As mentioned already above, the amoxicilloic acid concentration depleted much slower from the tissues than amoxicillin, both after p.o. ($t_{1/2AMO}$ = 4.5 h vs $t_{1/2AMA}$ = 8 h) and i.v. ($t_{1/2AMO}$ = 4 h vs $t_{1/2AMA}$ = 8 h) administration. The mean amoxicilloic acid concentration in all tissues was below the LOD at 72 h after cessation of medication. Because there is no significant influence of clavulanic acid on the amoxicilloic acid residue concentration in liver and kidney, a combined depletion curve for amoxicilloic acid in the tissues can be plotted. The residue concentrations of amoxicillin/clavulanic acid combination 36 h post treatment were combined with those of the amoxicillin administration alone. Linear regression analysis of the logarithmic transformed data can be considered for the calculation of the withdrawal periods. Using this approach, the withdrawal time was determined as the time when the one-sided, 95% upper tolerance limit of the regression line with a 95% confidence level was below the MRL (*30*). Generally, data below the LOQ can not be excluded from calculations, because they are due to real observations concerning the depletion kinetics. Therefore, the European Agency for the Evaluation of the Medicinal Products Guidelines recommends to set these data to one-half of the above fixed LOQ in the withdrawal time calculation. If however, the majority of data from one slaughter point is below the LOD or LOQ, the whole time point should be excluded (*30*). Using this approach, the withdrawal time for amoxicillin could only be calculated for kidney tissue after p.o. and i.v. administration: 1.52 and 1.46 days, respectively, resulting in a final withdrawal time of two days. This corresponds with the withdrawal time of the commercial amoxicillin formulation used in the present study (Amoxycillin 70%, KELA).

The withdrawal time of amoxicilloic acid is not possible to calculate, because the MRL of amoxicillin does not include the amoxicilloic acid metabolite. On the other hand, when the same MRL of amoxicillin should be used for the withdrawal time calculation, a withdrawal time of 3.59, 3.17, and 1.64 days for p.o. administration and 3.52, 2.62, and 2.85 days for i.v. dosing can be calculated for kidney, liver, and fat tissues, respectively. **Figure 3** illustrates a plot of withdrawal time calculation for amoxicilloic acid in porcine kidney after oral administration.

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In the case when amoxicilloic acid is used as marker, a final withdrawal time of four days should be obtained. The prolonged presence of the amoxicilloic acid residues therefore results in a doubling of the withdrawal time.

Discrepancies in Withdrawal Times by Different Analytical Methods. Some discrepancies were found in the literature with respect to the withdrawal time of amoxicillin in edible tissues of pigs. Martinez-Larrañaga et al. (*31*) found that after six days the mean amoxicillin concentration in porcine kidney and liver was below the MRL after oral administration of 20 mg kg^{-1} for five consecutive days. Another study in pigs reported that a withdrawal time of seven days in muscle and fat and four days in liver and kidney was necessary after oral administration of amoxicillin at 15 mg kg^{-1} for five consecutive days (*1*).

In a preliminary residue depletion study of amoxicillin in pigs, we calculated a withdrawal time of two days for amoxicillin after drinking water administration during five consecutive days (nonpublished data).

A possible explanation for the reported long withdrawal times of amoxicillin in the literature in comparison with those obtained in the present study could be due to differences in analytical methodology. The above-mentioned authors applied a fluorescence detection technique for amoxicillin in tissues after a precolumn derivatization of amoxicillin using formaldehyde in acidic media (*21*). In preliminary experiments it was observed that the fluorimetric detection method derivatizes amoxicillin as well as amoxicilloic acid into the same reaction product, with the same relative retention time in the chromatographic run (data not shown). This indicates that amoxicillin degrades into its amoxicilloic acid metabolite during the acid (trichloroacetic acid) tissue extraction, by opening of the β-lactam ring. In this case, the amount of parent compound concentration was overestimated. We also noticed that the amoxicilloic acid concentrations depleted much slower from the tissues than amoxicillin, so a much longer withdrawal time was calculated. The used extraction method during our experiments has, therefore, the advantage to determine all of the metabolites separately with a good specificity without over or underestimation of the amoxicillin concentration in tissues.

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