The biotransformation of sulfadimethoxine, sulfadimidine, sulfamethoxazole, trimethoprim and aditoprim by primary cultures of pig hepatocytes

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The *in vitro* biotransformation of three sulfonamides, trimethoprim and aditoprim, was studied using primary cultures of pig hepatocytes. Incubation of monolayer cultures with sulfadimethoxine (SDM), sulfamethoxazole (SMX) and ^{14}C-sulfadimidine (SDD) resulted in the formation of the corresponding *N*4-acetylsulfonamide to different extents, depending upon the molecular structure of the drug. Addition of the acetylsulfonamides to the cells showed that these compounds were deacetylated, each to a different extent. A relatively low degree of acetylation (in the case of SDD) was paralleled by extensive deacetylation (i.e. AcSDD), whereas extensive acetylation (i.e. SMX) was in concert with minor deacetylation (i.e. AcSMX). The addition of bovine serum albumin to the medium resulted in a decrease in conversion of sulfonamides as well as acetylsulfonamides. The main metabolic pathway of ^{14}C-trimethoprim (TMP) was *O*-demethylation with subsequent conjugation. Two hydroxy (demethyl) metabolites were formed, namely 3′- and 4′-demethyl trimethoprim, which were both glucuronidated while 3′-demethyl trimethoprim was also conjugated with sulphate. The capacity to form conjugates with either glucuronic acid or sulphate was at least as high as the capacity for *O*-demethylation since more than 90% of the metabolites were excreted as conjugates in the urine of pigs. Addition of ^{14}C-aditoprim (ADP) to the hepatocytes led to the *N*-demethylation of ADP to mono-methyl-ADP and di-desmethyl-ADP. During the incubation another three unknown ADP metabolites were formed. In contrast to TMP, no hydroxy metabolites or conjugated metabolites of aditoprim were formed. These *in vitro* results were in agreement with the *in vivo* biotransformation pattern of the studied sulfonamides and trimethoprim in pigs.

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INTRODUCTION

The use of isolated hepatocytes to study the *in vitro* biotransformation of drugs has been well established (Hoogenboom *et al.*, 1989; Fink-Gremmels & Van Miert, 1994; Van 't Klooster *et al.*, 1994b; Witkamp *et al.*, 1995; Monshouwer *et al.*, 1996). These *in vitro* studies can support and facilitate *in vivo* biotransformation studies thereby reducing the number of animals needed for *in vivo* experiments. Moreover, the use of radiolabelled drugs for *in vitro* biotransformation studies is convenient and not expensive, in contrast to *in vivo* studies of these drugs using large (food-producing) animals.

Sulfonamides and diaminopyrimidines are antimicrobial agents used in human and veterinary medicine for the treatment and prevention of bacterial infections (Van Miert, 1994). In Europe, sulfonamides are often combined with trimethoprim (TMP) because of synergistic effects against a number of both gram-positive and gram-negative pathogens. Aditoprim (ADP) is a structural analogue of TMP and has different pharmacokinetic properties (Ludwig *et al.*, 1985; Jordan *et al.*, 1987; Knoppert *et al.*, 1988).

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Similarly, ADP potentiates sulfonamides against porcine pathogens (Mengelers *et al.*, 1990). The molecular structures of the investigated drugs are depicted in Fig. 1.

In pigs, the main metabolic pathway for most sulfonamides is supposed to be *N*-acetylation (Vree *et al.*, 1985a; Kinabo & Nielsen, 1986; Rehm *et al.*, 1986; Nouws *et al.*, 1989, 1991; Shimoda *et al.*, 1990)). The rate and extent of the *in vivo* acetylation of sulfonamides is dependent on the structure of the sulfonamide. Furthermore, it was shown that after administration of *N*4-acetyl sulfonamides to humans and food-producing animals the compounds were partly deacetylated to the parent sulfonamide (Vree *et al.*, 1985b; Nouws *et al.*, 1989; Shimoda *et al.*, 1990). The main metabolic pathway for TMP in pigs is *O*-demethylation with subsequent conjugation (Friis *et al.*, 1984; Gyrd-Hansen *et al.*, 1984; Nouws *et al.*, 1991). Two hydroxy metabolites are formed, 3′- and 4′-demethyl trimethoprim (also referred to as M4 and M1, respectively), which are glucuronidated and sulfated. So far, the biotransformation of ADP has not been described.

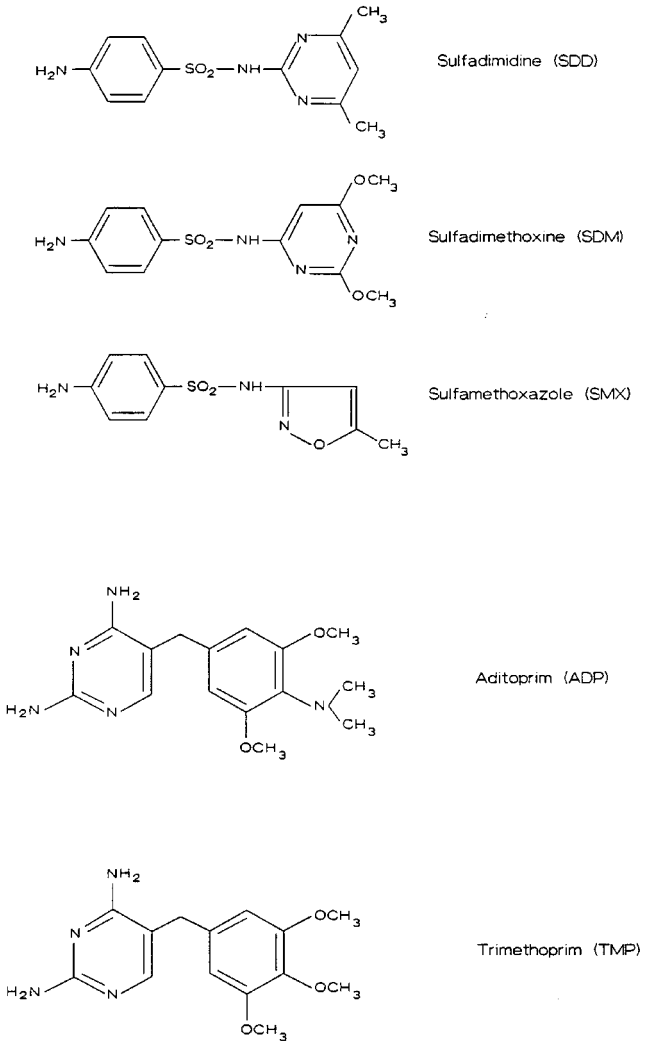

Fig. 1. Molecular structures of the investigated drugs.

The purpose of our study was to examine the overall *in vitro* biotransformation patterns of sulfadimethoxine (SDM), sulfadimidine (SDD), sulfamethoxazole (SMX), TMP and ADP by pig hepatocytes and to compare the results obtained with those from *in vivo* experiments. Special attention was given to the acetylation/deacetylation of sulfonamides/acetylsulfonamides and the demethylation of TMP and ADP. Conditions such as incubation time and drug concentration were varied and the influence of protein binding on the (de)acetylation of (acetyl)-sulfonamides was investigated. Radioactive labelled drugs, high-performance liquid chromatography (HPLC) and photo diode-array detection were used to verify the identity of metabolites. The application of column switching liquid chromatography for the direct analysis of the medium samples without prior extraction was investigated (Mengelers *et al.*, 1989).

MATERIALS AND METHODS

Chemicals and reagents

Williams' medium E (WME), foetal calf serum and penicillin/streptomycin were purchased from Flow Laboratories (Rickmansworth, UK). SDM, SDD, SMX, TMP, insulin, bovine serum albumin (BSA), arylsulphatase (*Helix pomatia*, type H-5), β-glucuronidase (*Escherichia coli*, 1000 U/vial) were obtained from Sigma (St. Louis, MO, USA). *N*4-acetyl SDM (AcSDM), *N*4-acetyl SDD (AcSDD) and *N*4-acetyl SMX (AcSMX) were a gift from Dr T.B. Vree (St. Radboud hospital, Nijmegen); 4′-demethyl TMP (M1), 3′-demethyl TMP (M4), ADP, mono-methyl ADP (mm-ADP), di-desmethyl ADP (ddm-ADP), 3′-demethyl ADP (3′-OH-ADP), radioactive labelled TMP (^{14}C-TMP, 1.59 MBq/mg) and ADP (^{14}C-ADP, 0.35 MBq/mg) were kindly donated by Dr W.F. Rehm (Hoffmann-La Roche, Basel, Switzerland). Radioactive labelled SDD (^{14}C-SDD, 4.1 GBq/mmol) was obtained from Amersham (Amersham, UK). Tissue culture dishes (24-wells dishes) were purchased from Costar (Badhoevedorp, The Netherlands). Ultrafiltration was carried out with the Amicon Micropartition System MPS1 (Grace Japan Co. Ltd, Tokyo, Japan). Triethylamine (TEA) was obtained from Fluka Chemie AG (Buchs, Switzerland) and all other chemicals were obtained from Merck AG (Darmstadt, FRG).

Animals

Seven to nine month-old sows in early pregnancy (7–10 days), used in fertility programs of the Department of Animal Husbandry of the Agricultural University at Wageningen, were slaughtered and the livers were treated as previously described (Hoogenboom *et al.*, 1989). Animals were not treated with veterinary drugs or feed additives for at least one month prior to slaughtering. The presented data are based on studies with hepatocytes isolated from livers of six animals. The results of each experiment were checked for reproducibility in at least one experiment using hepatocytes isolated from a control liver.

Preparation of cell cultures

The isolation of pig hepatocytes was carried out as described previously (Hoogenboom *et al.*, 1989). An aliquot of the final cell suspension was used to determine the number and fraction of trypan blue-excluding cells (Jaurequi *et al.*, 1981; Gyrd-Hansen *et al.*, 1984). Thereafter, the cell suspension was diluted with WME, supplemented with 5% foetal calf serum, insulin (0.5 μg/mL), penicillin (50 IU/mL) and streptomycin (50 μg/mL), to a density of 0.5×10^6–0.75×10^6 cells/mL. Subsequently, 0.5 mL of this suspension was transferred to each well in a multiwell dish and the hepatocytes were kept in 5% CO_2 at 38°C. After 4 h, cells were attached to the wall of the wells and the medium in each well was replaced by 0.25 mL supplemented WME. After overnight incubation the cells were washed with plain WME prior to the exposure to drugs.

Preparation of drug solutions

Stock solutions of drugs and metabolites were prepared in methanol (1.0 mg/mL). Standard solutions were prepared by drying aliquots of the stock solutions under a stream of nitrogen and dissolving the residues in WME by sonification. The standard solutions were sterilized by filtration (0.2 μm). Radioactive labelled drugs were dissolved in dimethyl sulfoxide (DMSO) (100 mM) and standard solutions were prepared by diluting the stock solutions in WME to a concentration of 50 or 100 μM. The final DMSO concentration in the medium was $\leqslant$ 0.1% (v/v).

Experimental design

After the cells were washed with WME, 0.5 mL of the standard solution was added to each multiwell and 0.25 mL was collected immediately after addition and snap-frozen ($t = 0$ h) in liquid nitrogen. After incubation, the remaining samples were treated similarly.

The biotransformation of 50 μM (acetyl)sulfonamide and 100 μM ^{14}C-SDD was studied after different incubation periods. The (de)acetylating capacity of the hepatocytes was checked with 50 μM (acetyl)sulfonamide for 96 h (four consecutive periods of 24 h). The influence of the substrate concentration on the (de)acetylation was studied after 24 h of incubation at 37°C with 10, 50 and 250 μM of (acetyl)sulfonamide. In order to determine the effect of protein binding on the (de)acetylation process, different (acetyl)sulfonamide concentrations (10, 50 and 250 μM) were added to the cells together with 500 μM BSA for 24 h. At the same time a control was run (24 h of incubation with 50 μM of (acetyl)sulfonamide without the addition of BSA). The influence of TMP on the biotransformation of sulfonamides was studied for different concentrations of TMP (0, 2, 10 and 50 μM) incubated together with 50 μM (acetyl)sulfonamides for 24 h.

The biotransformation of 10 μM TMP, 100 μM ^{14}C-TMP, and 100 μM ^{14}C-ADP was studied after different incubation times. The demethylating capacity of the hepatocytes was checked with 10 μM TMP and 10 μM ADP for 48 h (two consecutive periods of 24 h). The influence of the substrate concentration on the demethylation was studied after different incubation periods with 5, 10, 50 and 100 μM TMP and 10 and 50 μM ADP. The influence of the co-administration of sulfonamides on the biotransformation of TMP was studied for different concentrations of (acetyl)sulfonamides (0, 10, 50, 250 μM) incubated together with 10 μM TMP during 24 h. Deconjugation was carried out with samples collected at various incubation times.

Stability of drugs and metabolites in WME was investigated under the described incubation conditions after different periods.

Deconjugation

Deglucuronidation was carried out with a solution of β-glucuronidase (1000 U/mL) in 0.2 M potassium phosphate pH 6.8. Desulphatation was done with a solution of arylsulphatase (200 U/mL) in 0.2 M sodium acetate pH 5.0. Samples (0.1 mL) were mixed with 0.4 mL of the glucuronidase solution or 0.2 mL of the arylsulphatase solution. The mixtures were allowed to react for 2, 6 and 18 h at 38°C.

Analytical procedures

The analytical column used for the HPLC analysis of the (acetyl)sulfonamides was a Chromspher C18 column, 200 ′ 3.0 mm I.D. (Chrompack, Middelburg, The Netherlands). TMP, ADP and their metabolites were separated on a Supelcosil LC-18-DB HPLC column, 250 ′ 4.6 mm I.D. (Supelco Inc., Bellefonte, PA, USA). The column temperature was kept at 25°C. The applied mobile phases were mixtures of sodium acetate (NaAc) solutions, adjusted to the appropriate pH with acetic acid, and acetonitrile. For the analysis of TMP, ADP and their metabolites 0.2% TEA was added to the mobile phase to reduce tailing of the basic drugs and metabolites. The following eluents were used:

SDD: 0.05 M NaAc (pH 7.5): acetonitrile = 85: 15;
SDM: 0.05 M NaAc (pH 6.0): acetonitrile = 85: 15;
SMX: 0.05 M NaAc (pH 5.6): acetonitrile = 88: 12;
TMP: 0.05 M NaAc + 0.2% TEA v/v (pH 6.0):
acetonitrile = 85: 15;
ADP: 0.05 M NaAc + 0.2% TEA v/v (pH 7.5):
acetonitrile = 81: 19.

The eluents were filtered and degassed with helium before use, flow rates were 0.8 mL/min for (acetyl)sulfonamides or 1.0 mL/min (for TMP and ADP). The detection was performed at 270 nm (sulfonamides) or at 240 nm (diaminopyrimidines). The identity of parent drugs and known metabolites were confirmed with photo diode-array detection (HP 1041 A; Hewlett Packard).

Prior to analysis, samples were thawed and centrifuged for 5 min at 5000 ***g***. In the case of ADP, 0.1 mL supernatant was diluted with 0.4 mL 0.05 M NaAC + 0.2% TEA v/v (pH 7.5). The diluted sample was injected directly onto the analytical column. Supernatants (0.1 mL) containing (acetyl)sulfonamides or TMP were diluted with 0.4 mL 0.05 M NaAc, pH 4.6 or pH 7.0, respectively. The diluted samples were analysed without prior extraction by means of an on-line preconcentration technique (column switching HPLC) as described previously

(Mengelers *et al.*, 1989). Preconcentration was carried out with 0.05 M NaAc pH 4.6 (sulfonamides) or 0.05 M NaAc pH 7.0 (TMP).

HPLC eluents used in combination with an on-line radioactivity detector (LB 506C; Berthold, Wildbad, F.R.G.) did not contain buffer salts because of immiscibility with the scintillation liquid. The mobile phase for SDD was a mixture of water and acetonitrile (85:15) and the mobile phases for ADP and TMP were mixtures of 0.2% TEA (v/v), adjusted to the appropriate pH with acetic acid, and acetonitrile. Samples containing radioactive labelled compounds were centrifuged and diluted with an equal volume of the aqueous solution used for the preparation of the HPLC eluent and subsequent analysis was carried out by direct injection of these diluted samples.

In vitro *protein binding of (acetyl)sulfonamides*

In order to determine the *in vitro* protein binding of (acetyl)sulfonamides different substrate concentrations and 500 μM BSA were added to WME and kept for 24 h at the usual incubation conditions. The protein concentration applied corresponds to the actual albumin concentration in pig serum (Mitruka & Rawnsley, 1977). Subsequently, the unbound concentration of (acetyl)sulfonamides was determined after ultrafiltration (30 min at 2000 ***g***).

The influence of protein binding on the *in vitro* (de)acetylation of (acetyl)sulfonamides was determined after 24 h of incubation of hepatocytes with different substrate concentrations (10, 50 and 250 μM) and 500 μM BSA. The unbound concentration was determined after ultrafiltration and the total concentration was determined after liquid–liquid extraction. Samples (0.2 mL) were diluted with 1.0 mL sodium acetate buffer (0.1 M, pH 4.6) and extracted three times with 3 mL ethyl acetate. The combined extracts were dried under a stream of nitrogen and the residue was dissolved in 1.0 mL of preconcentration buffer before analysis.

RESULTS

In all experiments, hepatocytes were isolated from livers of adult sows with a yield varying between 2.5 and 10 million cells per gram wet weight. The fraction of cells excluding trypan blue varied between 90 and 95%. The hepatocytes readily attached themselves to uncoated culture dishes resulting in typical monolayer cultures.

Drugs and metabolites were not decomposed under the described experimental conditions.

Sulfonamides

After incubation of hepatocytes with ^{14}C-SDD it appeared that only AcSDD was formed. The HPLC analysis of samples incubated for different periods showed that there were no other metabolites formed (Fig. 2 A). The decrease in SDD as well as SDM and SMX was only due to *N*4-acetyl metabolite formation. The sulfonamides were acetylated to a different extent (Fig. 3 A), with AcSMX > AcSDM > AcSDD. Incubation with acetylsulfonamides revealed that the only enzymic conversion was deacetylation. Acetylsulfonamides were deacetylated each to a different extent (Fig. 3B), with SDD > SDM > SMX. A minor extent of acetylation (i.e. SDD) was paralleled by extensive deacetylation (i.e. AcSDD) whereas extensive acetylation (i.e. SMX) was in concert with minor deacetylation (i.e. AcSMX). With increasing incubation time the decrease in substrate concentration became less and the curves tended to flatten (Fig. 3). After the various incubation periods the total sulfonamide concentration (drug and metabolite) was equal to the added substrate concentration.

The (de)acetylating capacity of the hepatocytes was checked every 24 h during a period of 96 h. During the aging of the cultures the acetylating capacity increased whereas the deacetylation decreased. The increase in acetylation (for SDD, SDM and SMX 200%, 14%, and 118%, respectively) could not entirely be accounted for by a decrease in deacetylation (for AcSDD, AcSDM and AcSMX 20%, 30%, and 10%, respectively).

The *in vitro* protein binding of (acetyl)sulfonamides to BSA was determined at several concentrations after 24 h of incubation at 38°C (Table 1). The protein binding was quite constant over the investigated concentration range (5–250 μM). The difference in protein binding between a sulfonamide and its corresponding acetyl derivative was small although all three acetylsulfonamides were bound to a greater extent than their corresponding sulfonamides. The influence of protein binding on the *in vitro* biotransformation was determined at 3 different substrate concentrations after 24 h of incubation (Table 2). In general, the addition of BSA to the medium resulted in a lower conversion of acetylsulfonamides as well as sulfonamides. The reduced extent of (de)acetylation caused by the addition of BSA was more pronounced for those (acetyl)sulfonamides that showed a high protein binding, like SDM, AcSDM and AcSDD.

The influence of the substrate concentration on the conversions was determined after 24 h of incubation using 10, 50 and 250 μM of (acetyl)sulfonamide (Table 2). The acetylation decreased slightly, while the deacetylation increased slightly, with increasing concentrations.

There was no influence on the biotransformation of (acetyl)sulfonamides whenever trimethoprim was added to the medium (not shown).

Trimethoprim

The HPLC analysis of a sample incubated for 17 h with radioactive labelled TMP showed that polar compounds were formed (Fig. 2B). The sum of the radioactivity of these compounds accounted for 50.1% of the total radioactivity present in the sample. After deglucuronidation of the samples one of the two peaks in the chromatogram (called G) was converted to the demethylated metabolites M1 and M4. After desulfation the other peak (called S) was converted into mainly M4 and a little M1. Besides these conjugates, very small amounts of free demethylated derivatives were present. A small number of minor metabolites accounted for 2.3% of the total radioactivity

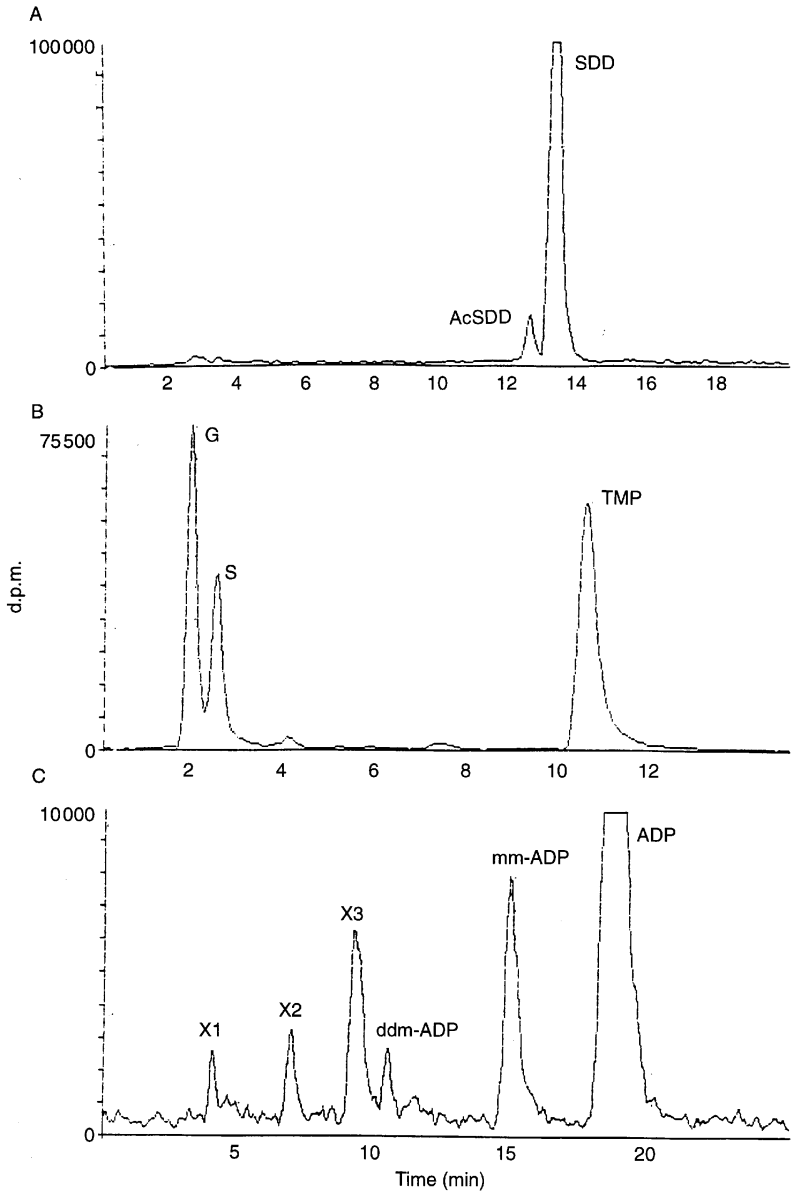

Fig. 2. HPLC chromatograms of medium samples after addition of SDD, TMP or ADP to pig hepatocytes. Hepatocytes were incubated with 100 μM of ^{14}C-SDD, ^{14}C-TMP or ^{14}C-ADP for 26 h, 17 h, and 25 h, respectively. *G* = Glucuronides of M1 and M4; S = Sulphates of M1 and M4; ddm-ADP = di-desmethyl ADP; mm-ADP = mono-methyl ADP; X1, X2, and X3 are unknown metabolites of aditoprim.

after 17 h of incubation. The formation of M1 and M4 (as glucuronides or as sulfates) after different incubation periods is shown in Fig. 4. The *O*-demethylating capacity of the hepatocytes was checked with 10 μM TMP for 48 h. With regard to the formation of M4 there was no difference in demethylation of TMP between the first 24 h and the second 24 h (total M4: 42–43%), whereas during the first and second day, respectively, 31% and 26% M1 was formed. The influence of the substrate concentration on the *O*-demethylation was studied after different incubation periods (Table 3). An increase in the substrate concentration resulted in a decrease of the *O*-demethylation reaction.

Co-administration of different concentrations of (acetyl)sulfonamides had no influence on the biotransformation of trimethoprim (not shown).

Aditoprim

The biotransformation of ADP was remarkably different from TMP. During the incubation of cells with 100 μM ^{14}C-ADP no. 3′-OH-ADP (an *O*-demethylated derivative) could be detected. Addition of ADP to the cells initially led to the *N*-demethylation of ADP to mm-ADP. In turn, this compound was slowly demethylated to ddm-ADP. Following prolonged incubation the concentration of mm-ADP and ddm-ADP reached a plateau and finally decreased slowly (Fig. 5). During the incubation of cells with ADP three unknown metabolites were observed (Fig. 2C). In increasing order of HPLC retention times they were referred to as X1, X2 and X3. The concentrations of these compounds increased until the end of the incubation. After 55 h of

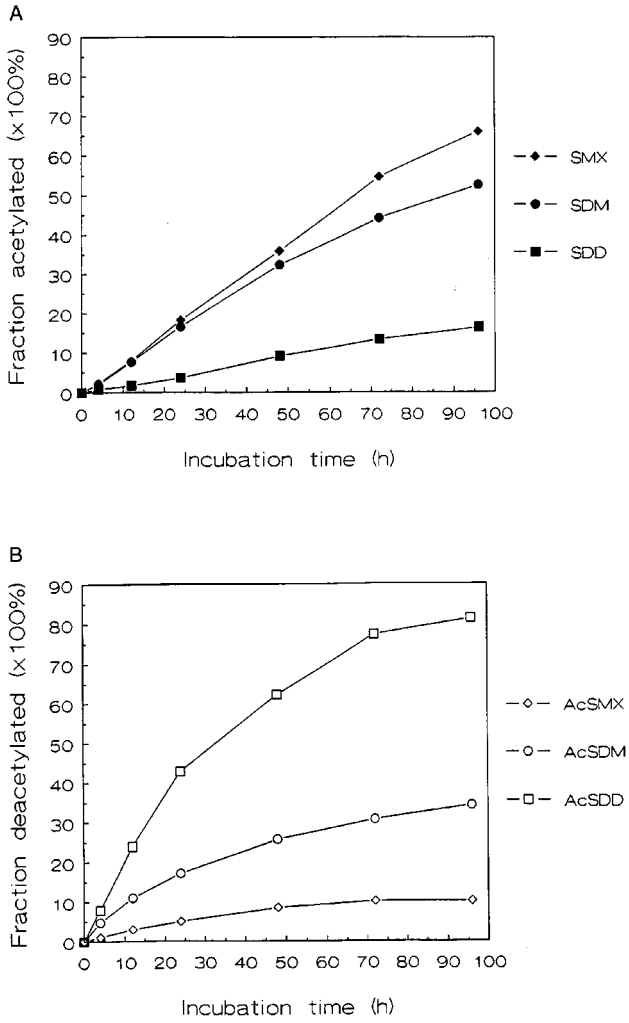

Fig. 3. The (de)acetylation of 50 μM (acetyl)sulfonamides after different incubation periods. The acetylated fraction of sulfonamides (A) and the deacetylated fraction of acetylsulfonamides (B) are expressed as the ratio of the 'metabolite' concentration to the total 'drug' concentration added to the medium. The values are the mean of two incubations with hepatocytes from different pigs. During each experiment duplicate multiwells were used and samples were analysed in duplo.

Table 1. The *in vitro* protein binding of several concentrations of (acetyl)sulfonamides to 500 μM BSA in WME after 24 h at 38°C and 5% CO_2

Substrate concentration	Percentage of (acetyl)sulfonamides bound *in vitro* to 500 μM BSA					
	SDM	AcSDM	SMX	AcSMX	SDD	AcSDD
5	99.1	99.5	67.1	72.0	80.4	92.8
10	99.5	99.4	64.1	67.0	83.9	93.5
25	98.0	99.2	65.0	67.6	85.8	92.2
50	98.2	99.1	64.7	68.4	84.8	93.8
100	97.2	98.8	61.0	65.3	83.5	91.5
250	95.3	98.5	62.1	64.8	80.9	89.5

incubation the radioactive fraction of the unknown compounds X1, X2 and X3 were 5.0%, 6.6% and 18.5%, respectively. After deconjugation of the samples with glucuronidase and/or sulfatase no other metabolites were detected in the chromatogram and no increase in any of the demethylated metabolites was observed.

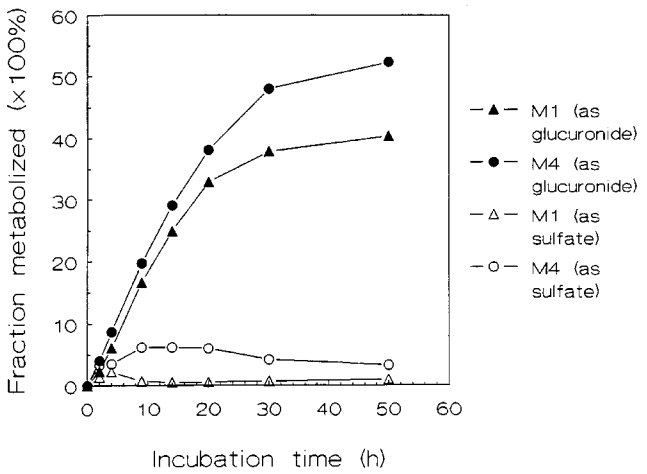

Fig. 4. The demethylation of 10 μM TMP after different incubation periods. The demethylated fraction is expressed as the ratio of the metabolite concentration (after deglucuronidation and desulfatation) to the total drug concentration added to the medium. The values are the mean of two incubations with hepatocytes from different pigs. During each experiment duplicate multiwells were used and samples were analysed in duplo.

Table 2. The (de)acetylated fraction of (acetyl)sulfonamides at different substrate concentrations (10, 50 and 250 μM) in the absence (−) or presence (+) of 500 μM BSA, after 24 h of incubation (incubation conditions: 38°C and 5% CO_2)

Substrate	500 μM BSA	Fraction metabolized* (×100%) at different substrate concentrations		
		10	50	250
SDM	−	17.2	17.9	17.0
SDM	+	4.7	5.0	5.8
SMX	−	22.1	20.5	18.3
SMX	+	10.8	14.2	12.7
SDD	−	5.8	4.4	5.4
SDD	+	1.9	2.1	2.0
AcSDM	−	11.1	16.5	19.5
AcSDM	+	2.3	4.4	4.6
AcSMX	−	3.3	6.0	6.3
AcSMX	+	0.9	1.9	2.7
AcSDD	−	41.3	43.5	44.6
AcSDD	+	11.4	11.9	14.0

*The (de)acetylated fraction is expressed as the ratio of the 'metabolite' concentration to the total 'drug' concentration added to the medium. The values are the mean of two incubations with hepatocytes from different pigs. During each experiment duplicate multiwells were used and samples were analysed *in duplo*.

The ADP and mm-ADP *N*-demethylating capacity of the hepatocytes was checked for 48 h (two incubation intervals). During the first 24 h the levels of mm-ADP and ddm-ADP were slightly higher (5–10%) than during the second 24 h interval.

The influence of the substrate concentration (10 and 50 μM

Table 3. The demethylated fraction of TMP (× 100%) at different substrate concentrations and after different incubation periods (incubation conditions: 38°C and 5% CO_2)

Metabolite	Incubation period (h)	Fraction demethylated* (× 100%) at different substrate concentration 5	10	50	100
M1	2	5.2	3.8	2.6	1.8
	4	9.2	8.4	6.5	4.6
	9	20.5	17.2	13.7	10.2
M4	2	9.5	7.3	4.3	3.2
	4	13.7	12.1	9.1	6.2
	9	29.1	25.9	17.6	13.6

*The metabolized fraction is expressed as the ratio of the total metabolite concentration (after deglucuronidation and desulfation) to the total drug concentration added to the medium. The values are the mean of two incubations with hepatocytes from different pigs. During each experiment duplicate multiwells were used and samples were analysed *in duplo*.

ADP) on the *N*-demethylation of ADP was studied after different incubation periods (Fig. 5B). The increase in substrate concentration resulted in a slower decline in the concentration of mm-ADP and ddm-ADP after maximum concentrations were reached, at 8 h and 20 h, respectively.

DISCUSSION

Biotransformation of SDM, SMX, and SDD in pig hepatocytes resulted in the formation of *N*4-acetyl metabolites, while no other metabolites could be detected. This is in agreement with results from *in vivo* studies showing that the main metabolic pathway for these sulfonamides in pigs is *N*-acetylation (Nouws *et al.*, 1989, 1991; Shimoda *et al.*, 1990; Mengelers *et al.*, 1995). Deacetylation of the investigated acetylsulfonamides was also observed *in vivo* after intravenous administration of AcSDM and AcSDD to pigs (Nouws *et al.*, 1989; Shimoda *et al.*, 1990; Mengelers *et al.*, 1995). However, despite the extensive *in vitro* deacetylation of AcSDD, urinary recovery of AcSDD was high because the renal clearance of AcSDD was 10 times higher than that of the parent drug (Nouws *et al.*, 1989). Although pig hepatocytes possess *O*-demethylating capacity, as was shown for TMP, sulfadimethoxine was not demethylated. This might be due to different iso-enzymes or to different affinities of these compounds for the same enzyme. At present it is not known whether acetylation and deacetylation proceed via the same enzyme system simultaneously or via two separate systems (Vree *et al.*, 1985a).

The percentage of (de)acetylation, based on the total (acetyl)sulfonamide concentration, was reduced after the addition of 500 μM BSA to the medium. This can be explained by a smaller extracellular fraction of free drug (and metabolite) available for uptake and subsequent biotransformation. Thus media applied for *in vitro* studies which are routinely enriched with serum might have an effect on the overall extent of biotransformation of xenobiotics by influencing free concentrations of substrates.

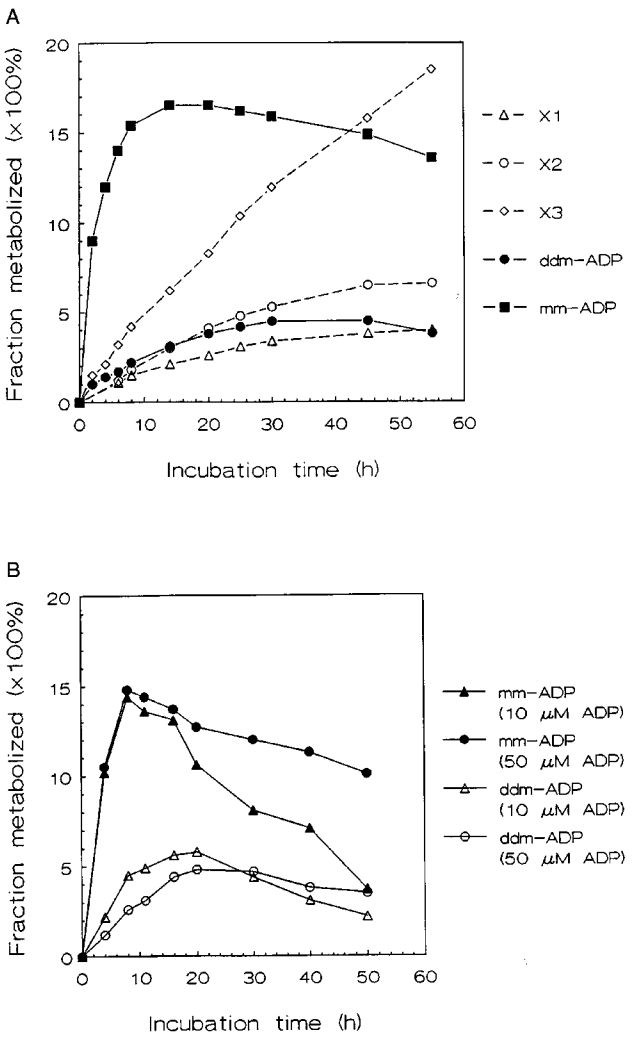

Fig. 5. The biotransformation of different concentrations of ADP after different incubation periods. The metabolized fraction of 100 μM ^{14}C-ADP (*A*) and of 10 and 50 μM ADP (*B*) after different incubation periods is expressed as the ratio of the metabolite concentration to the total drug concentration added to the medium. The values are the mean of two incubations with hepatocytes from different pigs. During each experiment duplicate multiwells were used and samples were analysed in duplo.

The major *in vitro* metabolic pathway for trimethoprim was *O*-demethylation with subsequent conjugation. *in vitro* sulfation and extensive glucuronidation of the demethylated derivatives M1 and M4 by hepatocytes corresponded well with the *in vivo* biotransformation of TMP in pigs (Friis *et al.*, 1984; Gyrd-Hansen *et al.*, 1984; Mengelers *et al.*, 1995). Similar results were obtained in both *in vitro* studies with suspensions of isolated pig hepatocytes and *in vivo* metabolism studies of TMP in pigs has already been shown by Gyrd-Hansen *et al.* (1984).

The elimination half-life of ADP in pigs is ≈ 8 h, whereas the half-life of TMP is 2.5–3 h (Nielsen & Rasmussen, 1975; Ludwig *et al.*, 1985; Jordan *et al.*, 1987). This may be due to the fact that ADP is not metabolized to polar compounds with high renal clearance rates, like the glucuronidated O-demethyl derivatives of TMP (Van 't Klooster *et al.*, 1994a). The reason why ADP is

not *O*-demethylated and subsequently glucuronidated is unclear. Physicochemical properties (pK_a and lipophilicity) are different for ADP and TMP. If the phenyl moiety of these compounds is important for the affinity of the drug to the *O*-demethylating enzyme, then the presence of a dimethylamino group might induce a steric hindrance for this enzyme. Analogous to the metabolism of TMP in animals (Friis *et al*., 1984; Gyrd-Hansen *et al*., 1984; Rehm *et al*., 1986), the three unknown metabolites of ADP might be derivatives obtained after α-*C*-oxidation and *N*-oxidation. The *in vitro N*-demethylation of ADP resembles the *in vivo N*-demethylation of baquiloprim in cattle (Tait *et al*., 1991). Baquiloprim [2,4-diamino-5-(8-dimethylamino-7-methyl-5-quinolylmethyl)pyrimidine] is another novel diaminopyrimidine derivative with a longer elimination half-life than TMP in food-producing animals (White *et al*., 1993; Dassanayake & White, 1994; Davies & MacKenzie, 1994; Lewicki *et al*., 1995). The results from the study performed by Tait *et al.* (1991). suggest that the *N*-demethylation of baquiloprim and *N*-desmethyl baquiloprim in calf is not reversible. Baquiloprim was extensively metabolized in cattle but other metabolites were not identified.

Finally, the co-administration of sulfonamides and TMP did not have a mutual influence on the *in vitro* biotransformation of either one of these compounds. The pharmacokinetic and therapeutic compatibility of TMP and sulfonamides has already been shown in various animal species and in man (Mandell & Sande, 1980; Prescott & Baggot, 1988). Because SDM is not *O*-demethylated *in vitro* by porcine hepatocytes, no competition between SDM and TMP for converting enzymes can be expected.

In conclusion, this study shows that the use of hepatocytes isolated from target animals for *in vitro* biotransformation studies of sulfonamides and diaminopyrimidines are a helpful tool in elucidating biotransformation patterns. Comparison between structural analogues revealed remarkable differences in the extent of biotransformation of sulfonamides and in the overall biotransformation pathways of diaminopyrimidines.

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REFERENCES

Dassanayake, L. & White, G. (1994) Administration of a bolus formulation of baquiloprim and sulphadimidine to calves: plasma concentration-time profiles and efficacy in suppressing experimental pneumonic pasteurellosis. *Veterinary Microbiology*, **38,** 255–262.

Davies, A.D. & MacKenzie, N.M. (1994) Pharmacokinetics of baquiloprim and sulphadimidine in pigs after intramuscular administration. *Research in Veterinary Science*, **57,** 69–74.

Fink-Gremmels, J. & Van Miert, A.S.J.P.A.M. (1994) Veterinary drugs: disposition, biotransformation and risk evaluation. *The Analyst*, **119,** 2521–2528.

Friis, C., Gyrd-Hansen, N., Nielsen, P., Nordholm, L. & Rasmussen, F. (1984) Pharmacokinetics and metabolism of trimethoprim in neonatal and young pigs. *Pediatric Pharmacology*, **4,** 231–238.

Gyrd-Hansen, N., Friis, C., Nielsen, P. & Rasmussen, F. (1984) Metabolism of trimethoprim in neonatal and young pigs: Comparative *in vivo* and *in vitro* studies. *Acta Pharmacologica et Toxicologica*, **55,** 402–409.

Hoogenboom, L.A.P., Pastoor, F.J.H., Clous, W.E., Hesse, S.E. & Kuiper, H.A. (1989) The use of porcine hepatocytes for biotransformation studies of veterinary drugs. *Xenobiotica*, **19,** 1207–1219.

Jaurequi, H.O., Hayner, N.T., Driscoll, J.L., Williams-Holland, R., Lipsky, M.H. & Galletti, P.M. (1981) Trypan Blue uptake and lactate dehydrogenase in adult rat hepatocytes – freshly isolated cells, cell suspensions, and primary monolayer cultures. *In Vitro*, **17,** 1100–1110.

Jordan, J.C., Klatt, P. & Ludwig, B. (1987) Pharmacokinetics of aditoprim, a new long-acting dihydrofolate reductase inhibitor, in heifers. *Journal of Veterinary Medicine A*, **34,** 33–41.

Kinabo, L.D.B. & Nielsen, P. (1986) Sulfatroxazole: pharmacokinetics, metabolism and urinary excretion in goats and pigs. *Journal of Veterinary Pharmacology and Therapeutics*, **9,** 55–62.

Knoppert, N.W., Nijmeijer, S.M., van Duin, C.T.M., Korstanje, C., van Gogh, H. & Van Miert, A.S.J.P.A.M. (1988) Some pharmacokinetic data of aditoprim and trimethoprim in healthy and tick-borne fever infected dwarf goats. *Journal of Veterinary Pharmacology and Therapeutics*, **11,** 135–144.

Lewicki, J., Maas, R.F.M., Witkamp, R.F. & Van Miert, A.S.J.P.A.M. (1995) Oral bioavailability and pharmacokinetics of baquiloprim in dwarf goats. *Research in Veterinary Science*, **58,** 268–271.

Ludwig, B., Jordan, J.C., Rehm, W.F. (1985) Pharmacokinetics of aditoprim, a new dihydrofolate reductase inhibitor, in pig and calf. In *Proceedings of the 3rd EAVPT Congress, Ghent, Belgium, August 25–29. 1985*. Eds. Van Miert, A.S.J.P.A.M. *et al.* pp. 114. EAVPT, Utrecht.

Mandell, G.L. & Sande, M.A. (1980) Antimicrobial agents. In *The Pharmacological Basis of Therapeutics*, 6th edn. Eds Gilman, A.G., Goodman, L.S. & Gilman, A, pp. 1106–1119. Macmillan Publishing Co., New York.

Mengelers, M.J.B., van Klingeren, B. & Van Miert, A.S.J.P.A.M. (1990) *In vitro* susceptibility of some porcine respiratory tract pathogens to aditoprim, trimethoprim, sulfadimethoxine, sulfamethoxazole, and combinations of these agents. *American Journal of Veterinary Research*, **51,** 1860–1864.

Mengelers, M.J.B., Oorsprong, M.B.M., Kuiper, H.A., Aerts, M.M.L., van Gogh, E.R. & Van Miert, A.S.J.P.A.M. (1989) Determination of sulfadimethoxine, sulfamethoxazole, trimethoprim and their main metabolites in porcine plasma by column switching HPLC. *Journal of Pharmaceutical and Biomedical Analysis*, **7,** 1765–1776.

Mengelers, M.J.B., Van Gogh, E.R., Kuiper, H.A., Pijpers, A., Verheijden, J.H.M. & Van Miert, A.S.J.P.A.M. (1995) Pharmacokinetics of sulfadimethoxine and sulfamethoxazole in combination with trimethoprim after intravenous administration to healthy and pneumonic pigs. *Journal of Veterinary Pharmacology and Therapeutics*, **18,** 243–253.

Mitruka, B.M. & Rawnsley, H.M. (1977) Pigs. In *Clinical biochemical and hematological reference values in normal experimental animals*. Eds Mitruka, B.M. & Rawnsley, H.M., pp. 160–164. Masson Publishing USA, Inc, New York.

Monshouwer, M., Witkamp, R.F., Nijmeijer, S.M., van Amsterdam, J.G. & Van Miert, A.S.J.P.A.M. (1996) Suppression of cytochrome P450- and UDP glucuronosyl transferase-dependent enzyme activities by proinflammatory cytokines and possible role of nitric oxide in primary cultures of pig hepatocytes. *Toxicology and Applied Pharmacology*, **137,** 237–244.

Nielsen, P. & Rasmussen, F. (1975) Half-life and renal excretion of trimethoprim in swine. *Acta Pharmacologica et Toxicologica*, **36,** 123–131.

Nouws, J.F.M., Mevius, D., Vree, T.B. & Degen, M. (1989) Pharmacokinetics and renal clearance of sulphadimidine, sulphamerazine and sulphadiazine and their *N*4-acetyl and hydroxy metabolites in pigs. *The Veterinary Quarterly.*, **11,** 78–86.

Nouws, J.F.M., Vree, T.B., Degen, M. & Mevius, D. (1991) Pharmacokinetics of a sulphamethoxazole/trimethoprim formulation in pigs after intravenous administration. *The Veterinary Quarterly.*, **13,** 148–154.

Prescott, J.F. & Baggot, J.D. (1988) Sulfonamides, Trimethoprim and Their Combination. In *Antimicrobial Therapy in Veterinary Medicine*. Eds Prescott, J.F. & Baggot, J.D., pp. 235–256. Blackwell Scientific Publications, Boston.

Rehm, W.F., Teelmann, K. & Weidekamm, E. (1986) General aspects of metabolism, residues and toxicology of sulfonamides and dihdrofolate reductase inhibitors. In *Drug Residues in Animals*. Ed. Rico, A.G., pp. 65–109. Academic Press, Inc, Orlando.

Shimoda, M., Vree, T.B., Beneken-Kolmer, E.W.J. & Arts, Th.H.M. (1990) The role of plasma protein binding on the metabolism and renal excretion of sulphadimethoxine and its metabolite *N*4-acetylsulphadimethoxine in pigs. *The Veterinary Quarterly*, **12,** 87–97.

Tait, A.J., Johnson, D.E. & White, G. (1991) Comparative pharmacokinetics of baquiloprim and two metabolites in the bovine. In *Proceedings of the 5th EAVPT Congress, Copenhagen, Denmark, August 18–22. 1991*. Eds. Friis, C. *et al. Acta Veterinaria Scandinavica, Supplementum*, **87,** 151–153.

Van Miert, A.S.J.P.A.M. (1994) The sulfonamide-diaminopyrimidine story. *Journal of Veterinary Pharmacology and Therapeutics*, **17,** 309–316.

Van 't Klooster, G.A.E., Maas, R.F., Csiko, G. & Van Miert, A.S.J.P.A.M. (1994a) Inhibition of glucuronidation activities by trimethoprim analogues in primary cultures of cattle, goat and rat hepatocytes. In Proceedings of the 6th EAVPT Congress, Edinburgh, UK, August 7–11 (1994) Ed. Lees., P. Oxford, Blackwell Scientific Publications, pp. 210–211.

Van 't Klooster, G.A.E., Woutersen-van Nijnanten, F.M.A., Blaauboer, B.J., Noordhoek, J. & Van Miert, A.S.J.P.A.M. (1994b) Applicability of cultured hepatocytes derived from goat, sheep and cattle in comparative drug metabolism studies. *Xenobiotica*, **24,** 417–428.

Vree, T.B., Hekster, Y.A., Nouws, J.F.M. & Dorrestein, G.M. (1985a) Pharmacokinetics of sulfonamides in animals. In *''Pharmacokinetics of sulfonamides revisited'', Antibiotics and Chemotherapy Vol. 34*. Eds Schönfeld H. & Hahn, F. E. pp. 130–170. Karger, New York.

Vree, T.B., Hekster, Y.A. & Tijhuis, M.W. (1985b) Metabolism of sulfonamides. In *Pharmacokinetics of sulfonamides revisited, Antibiotics and Chemotherapy Vol. 34*, Eds Schönfeld, H. & Hahn, F.E. pp. 5–121. Karger, New York.

White, G., Daluge, S.M., Sigel, C.W., Ferone, F. & Wilson, H.R. (1993) Baquiloprim, a new antifolate antimicrobial: *in vitro* activity and pharmacokinetic properties in cattle. *Research in Veterinary Science*, **54,** 372–378.

Witkamp, R.F., Nijmeijer, S.M., Monshouwer, M. & Van Miert, A.S.J.P.A.M. (1995) The antibiotic tiamulin is a potent inducer and inhibitor of cytochrome P4503 A via the formation of a stable metabolic intermediate complex. Studies in primary hepatocyte cultures and liver microsomes of the pig. *Drug Metabolism and Disposition*, **23,** 542–547.