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Isolation and structural elucidation of tiamulin metabolites formed in liver microsomes of pigs

Anne Kruse Lykkeberg *, Claus Cornett, Bent Halling-Sørensen, Steen Honoré Hansen

Department of Pharmaceutics and Analytical Chemistry, The Danish University of Pharmaceutical Science, Universitetsparken 2, DK-2100 Copenhagen, Denmark

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Abstract

Although the antimicrobial tiamulin is extensively metabolized in pigs, the metabolism is not well investigated. In this work the NADPH dependent metabolism of tiamulin in liver microsomes from pigs has been studied. The tiamulin metabolites formed in the incubations were analysed using LC–MS, and three major metabolites were isolated using solid phase extraction and preparative HPLC. The final structure elucidations were performed by tandem mass spectrometry and ^{1}H and ^{13}C NMR. The structures of the metabolites were found to be 2β-hydroxy-tiamulin, 8α-hydroxy-tiamulin and *N*-deethyl-tiamulin. In addition, the LC–MS chromatograms revealed two other minor metabolites. From their chromatography and from MS^2 analysis the structures were estimated to be 2β-hydroxy-*N*-deethyl-tiamulin and 8α-hydroxy-*N*-deethyl-tiamulin, but the structures were not confirmed by NMR. In these studies approximately 20% of tiamulin was deethylated, 10% was hydroxylated in the 2β-position and 7% was hydroxylated in the 8α-position. About 40% of tiamulin was metabolized during the incubation conditions used.

The protein precipitation in the incubations was performed using perchloric acid, and the preparative purification was performed under alkaline conditions. Therefore, the stability of the metabolites under these conditions was studied. The metabolites were found to be stable in the acid solution, but under alkaline conditions, particularly at room temperature, the stability of especially 8α-hydroxy-tiamulin was considerably reduced (40% loss after 1 week).

Keywords: Tiamulin; *N*-deethyl-tiamulin; Metabolism; Liver microsomes; Swine; LC–MS; NMR

1. Introduction

Tiamulin is an antimicrobial widely used in the treatment and prophylaxis of dysentery and pneumonia in pigs and poultry. In 2004 the tiamulin consumption in Denmark was about 8 t, primarily used for pigs. Tiamulin is a semisynthetic drug developed from the antibiotic pleuromutilin isolated from the basidiomycete fungi *Pleurotus* or *Clitopilus* [1]. Tiamulin and valnemulin are the only pleuromutilin derivates on the market; both drugs are only used in veterinary practice. The chemical structures of valnemulin, tiamulin and pleuromutilin are shown in Fig. 1.

In 2004, approximately 25 millions pigs were produced in Denmark, which is equivalent to five times the population [2]. However, this production results in a lot of manure as a side-product and the manure will often be applied to the fields. Furthermore antimicrobials and residues of antimicrobials are transmitted to the fields with the manure slurry [3]. It is therefore relevant to know which antimicrobial metabolites are generated in the pigs and excreted in the manure. The antimicrobial and maybe also the metabolites may influence the bacteria in the environment. The potency of the metabolites on tiamulin sensitive and resistant bacteria and on environmental relevant bacteria will be investigated in a future work.

Tiamulin was patented in London in 1978 [4], and the metabolism of the compound is poorly investigated in spite of the historic age of the antimicrobial. No structures of tiamulin metabolites have been published in the literature so far. According to a rapport of the European Agency for the Evaluation of Medicinal Products (EMEA) [5] more than 15 tiamulin metabolites have been detected in the liver of pigs orally dosed with tiamulin hydrogen fumarate. No single metabolite accounted for more than 7% of the total residue of tiamulin in urine, faeces and bile.

* Corresponding author. Tel.: +45 35 30 60 00/62 68; fax: +45 35 30 60 10.
E-mail address: ak@dfuni.dk (A.K. Lykkeberg).

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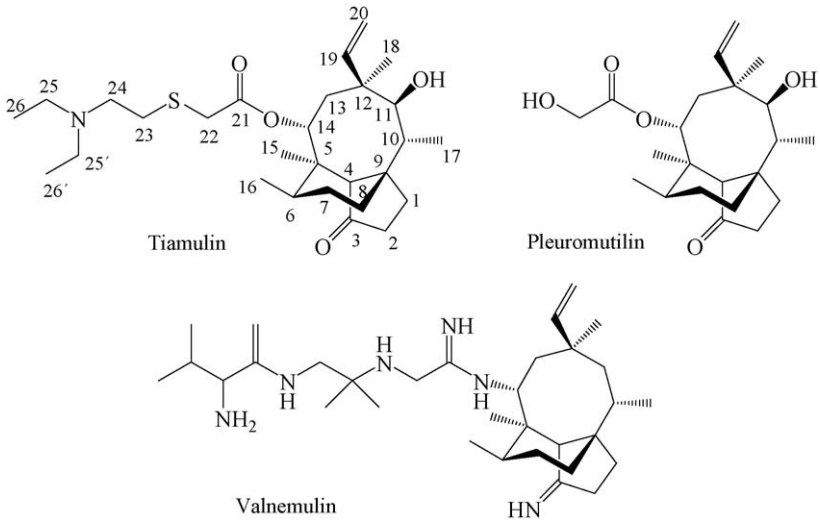


Fig. 1. Chemical structures of tiamulin, valnemulin and pleuromutilin.

According to Berner et al. [6] pleuromutiline and the derivates of pleuromutiline are extensively metabolically hydroxylated in the 1β-, 2β- and 8α-positions. It is therefore likely, that tiamulin is also hydroxylated in these positions.

The aim of this work was to investigate the NADPH dependent metabolism of tiamulin in liver microsomes from pigs. The metabolites were analysed using LC–MS and isolated using SPE and preparative HPLC-UV. The structures were elucidated using MS^2 and ^1H and ^{13}C NMR.

2. Experimental

2.1. Chemicals

Tiamulin hydrogen fumarate was purchased from Apodan (Løgstør, Denmark), formic acid, Tris(hydroxymethyl)aminomethane and sodium dihydrogenphosphate dihydrate were purchased from Merck (Darmstadt, Germany), ammonia-solution 28% was purchased from Merck eurolab (Briare, France), sucrose was purchased from Dansukker (Copenhagen, Denmark), β-Nicotinamide Adenine Dinucleotide Phosphate sodium salt, D-glucose-6-phosphate disodium salt hydrate and glucose-6-phosphate dehydrogenase from baker's yeast were purchased from Sigma (St. Louis, MO) and deuterium oxide was purchased from Aldrich Chemical Company (Milwaukee, WI, USA). All other chemicals were of reagent grade and HPLC quality.

2.2. Preparation of microsomes

The microsomes were prepared from the liver of a female Landrace/Yorkshire/Duroc pig with a weight of 23 kg. It received an intravenous injection of heparin immediately before the slaughtering to get a good bleeding of the animal [7]. The liver was stored for 0.5–1 h in icecold 0.1 M Tris-buffer, pH 7.4, until the preparation of the microsomes. A buffer containing 0.1 M Tris-buffer, pH 7.4, 0.25 M sucrose and 1 mM EDTA was used for homogenization and centrifugation using three parts of buffer to one part of tissue. The homogenization was first performed using a household handblender equipped with a chopper attachment to homogenize all large pieces of tissue. Hereafter, it was homogenized for 0.5–1 min using a DIAX 600 homogenizer (Heidolph, Germany). All homogenization was performed in a refrigerated room with a temperature of 4 °C. The homogenate was centrifuged in a Beckman/Optimum LE-80 Ultracentrifuge with a rotor Ti 42.1 (Münich, Germany) at 12,000 × *g* for 20 min at 4 °C to spin down nuclei, mitochondria and cellular debris. Supernatant from this step was centrifuged in the ultracentrifuge at 100,000 × *g* for 60 min at 4 °C. The resulting pellet was rinsed with 0.1 M potassium phosphate, pH 7.4, and it was resuspended in the same buffer in a volume corresponding to the volume of the liver tissue. The protein concentration was determined by the colometric method of Lowry et al. [8].

2.3. In vitro incubation

The incubation mixture consisted of 0.1 M phosphate buffer (pH 7.4), an NADPH-generating system (0.8 mM β-$NADP^+$, 5 mM glucose 6-phosphate and 1 U/ml glucose 6-phosphate dehydrogenase) and the liver microsomes (2 mg protein/ml). The incubation mixture was preincubated in the presence of the NADPH-generating system at 37 °C for 3 min, and the reaction was initiated by addition of the substrate (tiamulin hydrogen fumarate, 0.5 M) and continued for another 60 min. The total volume was 500 μl for the analysis and 52.5 ml for the preparative isolation. The reaction was terminated by the addition of perchloric acid (to a concentration of ca. 2.5%) or 50% acetonitrile. It was kept on ice for 5 min and at room temperature for 5 min and then centrifuged (Heraeus, Sepatech, Labofuge 200, Osterode, Germany) at 2000 × *g* for 10 min to precipitate the proteins. The supernatant was analysed or used for isolation of the metabolites.

To evaluate the performance of the in vitro incubations, testosterone was incubated in the microsomes added the NADPH generating system.

In the incubations used for isolation of the metabolites the proteins were precipitated by the addition of perchloric acid. The stability of the metabolites in the supernatant was studied under these acidic conditions. After protein precipitation and centrifugation different samples of the supernatant containing the metabolites were exposed to the following: 1.5 weeks at room temperature, 2.5 weeks at room temperature, three freeze-thaw cycles and five freeze-thaw cycles, respectively. Hereafter the samples were frozen at −20 °C. The degradation of the metabolites was compared to samples, which were frozen immediately after protein precipitation and centrifugation. All the samples were analysed using LC–MS.

2.4. Analytical HPLC-system

An Agilent 1100 HPLC gradient system (Agilent Technologies, Palo Alto, CA, USA) equipped with an auto sampler, a pump and a thermostated column oven (operated at 35 °C) was used. The analytical column was an Xterra MS (Waters Corporation, Milford, MO, USA) C_{18} chromatographic column (100 mm × 2.1 mm I.D., 3.5 μm).

The separation was performed using gradient elution. Mobile phase A: acetonitrile-formic acid (pH 2.75; 0.02 M) (5:95, v/v) and mobile phase B: acetonitrile-formic acid (pH 2.75; 0.02 M) (95:5, v/v).

The gradient was as follows: 0–1 min 10% B, 1–10 min a linear gradient to 50% B, 10–13 min 50% B, 13–14 min a linear gradient back to 10% B. The analysis time was 20 min. The injection volume was 5.0 μl and the flow rate 0.25 ml/min.

The testosterone metabolism was analysed by the use of a slight modification of the method of Magnusson and Sandström [9] using a shorter column. The column was a Zorbax SB-CN column (150 mm × 4.6 mm I.D, 5 μm), and the column temperature was 37 °C. Mobile phase A: 33% methanol with 0.5 mM ammonium acetate added and mobile phase B: 55% methanol with 0.5 mM ammonium acetate added. The following gradient was used: 0–12 min: 0–32% B and 12–21 min: 32–100% B. The injection volume was 10 μl, the flow rate was 1.0 ml/min and the UV-detection was performed operating at 240 and 254 nm.

2.5. LC–MS analysis

A Hewlett Packard series 1100 MSD single quadropole mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA) with an electro spray ionization interface operating in positive mode was used for quantification. Nitrogen was used as nebulizing gas at a pressure of 25 psi at 10 l/min, the temperature was 350 °C and the capillary voltage of 2000 V. The MS was scanned from *m/z* 460 to 515 using a fragmentor voltage of 80 V. Collection and treatment of data was done using ChemStation Rev. A.09.01 (Agilent Technologies).

MS^2-data were performed using an Agilent 1100 series LC-MSD ion trap mass spectrometer (Agilent, Waldbronn, Germany) equipped with an electro-spray ionization interface. Nitrogen was used as a nebulizing gas at a pressure of 40 psi at 10 l/min, the temperature was 350 °C and the capillary voltage 4.5 kV. The MS settings such as trap drive and skimmer voltage were optimized by infusion of a solution of tiamulin. A fragmentation voltage of 0.8 V was used for manual MS/MS analysis. MSD Trap Control Version 5.0 (Bruker Daltonik) and DataAnalysis Version 2.1 (Bruker Daltonik) were used to control the MSD trap and for data analysis, respectively.

2.6. Preparative isolation of metabolites

The metabolites were synthesized enzymatically as described earlier, now in batches of 52.5 ml. Proteins were precipitated using perchloric acid to avoid large amounts of acetonitrile to disturb the following solid phase extraction. A 25% ammonia-solution was added to the supernatant to a final concentration of 2%, and it was subsequently solid phase extracted through Oasis® HLB 6 cm^3 (0.2 g) extraction cartridges (Waters). The cartridges were conditioned with 2 ml acetonitrile, equilibrated with 2 ml 2% ammonia, loaded with 70 ml of the alkaline supernatant, washed with 1 ml 35% acetonitrile in 2% ammonia and eluted with 12 ml 35% acetonitrile in 2% ammonia. The 12 ml of eluate contained primarily the metabolites, and most of the tiamulin was restrained on the cartridges, and it could be eluted with 100% acetonitrile. The 12 ml of eluate was frozen at −80 °C and then freeze-dried in a Hetosicc CD52-1 freeze-dryer (Heto Lab equipment, Denmark).

The lyophilised extract was dissolved in 0.5 ml acetonitrile and added 0.5 ml water. Purification of the metabolites was performed using the following preparative HPLC-system:

A LC-10AD liquid chromatograph (Shimadzu, Duisburg, Germany) was used and the detection was performed with an SPD-10A UV-detector (Shimadzu, Duisburg, Germany) at 254 nm with a DP700 integrator (CE Instruments, Milan, Italy) connected. The preparative column was an Xterra MS (Waters Corporation, Milford, MO, USA) C_{18} chromatographic column (100 mm × 19 mm I.D., 5 μm). The operating flow rate was 5.0 ml/min. Mobile phase A: acetonitrile-ammonia (10 mM) (30:70, v/v) and mobile phase B: acetonitrile-ammonia (10 mM) (70:30, v/v). The following step-gradient was used: 0–19 min 50% B (elution of the metabolites) and 19–29 min 100% B (elution of residues of tiamulin). The column was equilibrated with 50% B before next injection. The metabolites were collected in vials added one drop of 6% hydrochloric acid. Each fraction was subsequently freeze-dried. The metabolites were purified a second time using the same HPLC procedure.

The stability of the metabolites in 2% ammonia at room temperature and at 5 °C was studied. A 25% ammonia-solution was added to the supernatant from the tiamulin incubations (protein precipitated with perchloric acid) to a final concentration of 2% ammonia. Samples were kept at room temperature and in a refrigerator, respectively, and then subsequently frozen at −20 °C after 0, 2, 4 h, 1, 2 days and 1 week, respectively. The samples were then analysed using LC–MS.

Table 1
Acquisition parameters for the NMR analysis

	^{1}H NMR	^{13}C NMR	COSY	NOESY	^{1}H-^{13}C COSY	HMBC
TD (F1)	–	–	512	512	512	256/512
TD (F2)	32768	65536	1024	2048	4096	4096
SI (F1)	–	–	1024	1024	1024	1024
SI (F2)	131072	32768	1024	1024	1024	1024
Pulses	60°	24°	90°	90°	42°	90°
AQ						
Tiamulin	5.40	1.36	0.17	0.34	0.68	0.68
Metabolites	2.94	1.25–1.36	0.09	0.18	0.37	0.34–0.37
NS						
Tiamulin	32	128	8	16	32	32
Metabolites	512	65536	16	32	96	112/128
SWH						
Tiamulin	3034.0	24038.5	3034.0	3034.0	3034.0	3034.0
Metabolites	5580.4	24038.5	5580.4	5580.4	5580.4	5580.4
LB						
Tiamulin	–	10	–[a]	–[b]	–	–
Metabolites	–	5	–[a]	–[b]	–	–
D1 (s)						
Tiamulin	1	2	2	2	2	2
Metabolites	1	2	2–4	2	1	1

TD: time domain data, SI: size of the real part of the spectrum, AQ: acquisition time, NS: number of scans, SWH: spectral width, LB: exponential line broadening applied prior to Fourier transformation, and D1: relaxation delay between successive scans/transients.
[a] Unshifted squared sine bell in both dimensions.
[b] Shifted squared sine bell in both dimensions.

2.7. NMR analysis

The NMR data of tiamulin and metabolite M1, M2 and M3 were acquired using an AMX-400 spectrometer (Bruker, Rheinstetten, Germany). ^{1}H NMR data were obtained at 400.13 MHz and ^{13}C NMR data at 100.6 MHz. All NMR spectra were recorded in D_2O. For the structural elucidation, the following spectra were obtained: ^{1}H NMR, ^{13}C NMR, COSY, NOESY, heteronuclear short range/one bond correlated (HMQC) and heteronuclear long range correlated (HMBC). Acquisition parameters are shown in Table 1.

3. Results and discussion

3.1. LC–MS analysis of tiamulin metabolites

The in vitro metabolism system was evaluated by incubation of testosterone in the microsomes. It was attempted to analyze the metabolites of testosterone using MS-detection, but it was not possible to get a sufficient ionization of the hydroxylated metabolites at the two mass spectrometers used in this work. Several hydroxylated testosterone metabolites were formed, and this is consistent with the metabolism of testosterone in swine liver microsomes found by Donato et al. [10]. This proves that the liver microsomes do contain enzymatic activity.

The tiamulin metabolites produced in the pig liver microsomes were initially analysed using LC–MS. The total ion chromatogram (TIC) is shown in Fig. 2a. The extracted ion chromatograms (EIC) of the ions 466.4, 510.4 and 482.4 are shown in Fig. 2b–d, respectively. The chromatograms illustrated with dotted lines correspond to a blank incubation, where tiamulin was incubated in the microsomes without the NADPH generating factor added. It is seen that tiamulin is extensively metabolized, since the area of tiamulin is reduced with around 40% in the chromatogram with cofactor added. Three metabolites are formed in large amounts, and two metabolites in smaller quantities. The metabolites and the compound with the *m*/*z*-value of 464 (Fig. 2a) are produced only in the presence of the NADPH generating system, which suggests that they were generated by the Cytochrome P450 system. Both metabolite M1 and M2 have an *m*/*z*-value of 510, which suggests that they are hydroxylated or alternatively oxidized at the nitrogen or the sulphur to form N-oxides or S-oxides. Metabolite M3 has an *m*/*z*-value of 466, which is 28 less than the *m*/*z*-value of tiamulin. This indicates a deethylation at the amino group. Metabolite M4 and M5 were produced only in small amounts. They have the *m*/*z*-value 482 corresponding to metabolites, which are both hydroxylated and deethylated. The compound with the *m*/*z*-value 492 is an impurity of tiamulin. It is probably 11-oxotiamulin, which is the only impurity of tiamulin hydrogen fumarate with this *m*/*z*-value mentioned in the European Pharmacopoeia [11]. The chromatogram shows that the impurity was metabolized, since the peak area was reduced when the cofactor was added. It is probably metabolized to the metabolite with the *m*/*z*-value 464, which could correspond to *N*-deethyl-11-oxotiamulin.

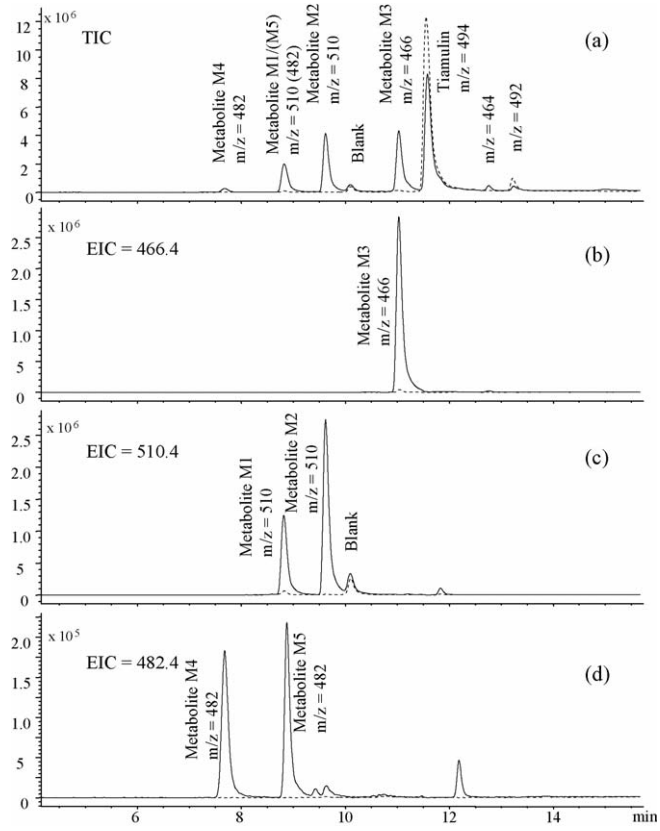


Fig. 2. Chromatogram of tiamulin incubated with pig liver microsomes. The chromatographic data are described in "Section 2.4 Analytical HPLC-system." The dotted chromatograms correspond to blank incubations, where tiamulin was incubated without the NADPH generating system added. (a) Total ion chromatogram, (b) extracted ion chromatogram of the mass 466.4, (c) extracted ion chromatogram of the mass 510.4 and (d) extracted ion chromatogram of the mass 482.4.

It is not possible to quantify the metabolites using the peak areas in the mass spectres, when the authentic reference substances are not available, since the ionization of the substances in the mass spectrometer may vary resulting in different response factors. The ionization of tiamulin and the metabolites may be expected to be different in spite of only small differences in the molecular structures. Tiamulin and the metabolites do not contain many chromophore groups, and they therefore exhibit a poor UV-absorption. It was therefore not possible to quantify the metabolites from UV-detection in these low concentrations neither.

3.2. Stability

After the incubation of tiamulin with microsomes, the proteins were precipitated using perchloric acid. The stability under these acid conditions was therefore investigated. The three metabolites were stable at room temperature, and no degradation was seen when the samples were exposed to freeze-thaw cycles.

The solid phase extraction and the preparative isolation of the metabolites had to be performed in alkaline solvents to prevent band broadening and tailing in the HPLC-system. Therefore, the stability of the metabolites in 2% ammonia was studied. Some of the samples were kept in a refrigerator (4 °C) and others at room temperature. The metabolites M1 and M3 were stable even after 1 week in the refrigerator. Metabolite M2 on the other hand started to degrade already after 1 day, and after 1 week around 15% of the metabolite had degraded.

The stability of the metabolites in alkaline solution is lower at room temperature. The degradation of metabolite M2 was already observed after four hours, and after 1 week at room temperature around 40% had degraded. The metabolites M1 and M3 also started to degrade, and after 1 week kept at room temperature around 10% of both metabolites had degraded.

These stability experiments showed that the tiamulin metabolites were most stable in acid solutions and the stability in alkaline solution was better in the refrigerator (4 °C) than at room temperature (25 °C). Therefore, the following procedures were developed for the isolation of the metabolites:

After protein precipitation and centrifugation the supernatant was made alkaline immediately before the solid phase extraction. The solution of the metabolites eluted in 35% acetonitrile in 2% ammonia were frozen at −80 °C immediately after elution. The lyophilised extract of tiamulin metabolites was dissolved immediately before injection into the preparative HPLC-system, and the solution were kept in a refrigerator or on ice between the injections. The fractions containing the metabolites were acidified immediately after separation and frozen as soon as possible.

3.3. Isolation of the metabolites

An initial purification of the large-scale microsome preparations containing the generated metabolites was performed using SPE. The proteins in the incubation mixture were precipitated using perchloric acid instead of acetonitrile to avoid organic solvents in the extractions columns.

In Oasis® HLB (0, 2 g) extraction cartridges the tiamulin metabolites were eluted before tiamulin using 35% acetonitrile in 2% ammonia-solution. In the first ml no metabolites were eluted, but the eluate was strongly yellow indicating the presence of impurities. In the following 12 ml of eluate almost all the metabolites were eluted, and the solution contained only small amounts of tiamulin. These 12 ml were freeze-dried and was then ready for preparative HPLC.

In the first attempt to develop a preparative HPLC-system the analytical HPLC method with mobile phases containing 20 mM formic acid was scaled directly up to the preparative HPLC-column. This resulted in broadening of the bands in the HPLC-system of especially tiamulin and *N*-deethyl-tiamulin. It was not possible to load more than 0.5 mg before the retention time of tiamulin was changed. Therefore, pH was increased to above the pK_a-level, and a chromatographic method using mobile phases containing 10 mM ammonia was developed. In this system it was possible to get sharp peaks and to load at least 5–10 mg of metabolites.

A typical chromatogram is shown in Fig. 3. The retention factors k' were: metabolite M2: 7.6, metabolite M1: 10.2, metabolite M3: 11.0 and tiamulin: 18. The fractions containing the metabolites were collected and freeze-dried. To obtain a sufficient purity

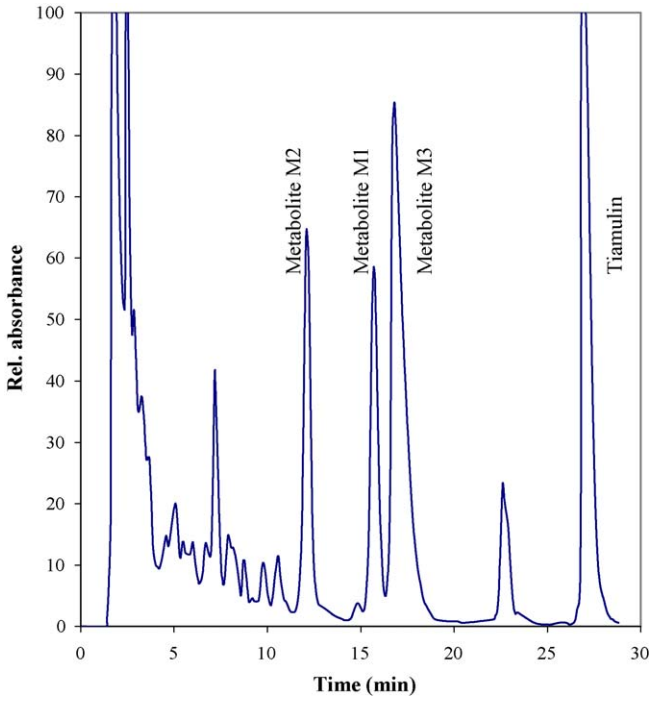


Fig. 3. Chromatogram of tiamulin metabolites injected into the preparative HPLC-system. Column: Xterra MS C_{18} chromatographic column (100 mm × 19 mm I.D., 5 μm), flow 5.0 ml/min, *Mobile* phase: acetonitrile/10 mM ammonia by following step-gradient: 0–19 min 50% acetonitrile and 19–27 min 70% acetonitrile and UV = 254 nm.

of the metabolites the collected fractions were purified a second time using the same chromatographic system.

The metabolites exhibit a very poor UV-absorption, but in these high concentrations used it is possible to detect them using an UV-detector. It is seen from the areas of the three peaks corresponding to the metabolites, that *N*-deethyl-tiamulin account for 54% of the three metabolites and 2β-hydroxy-tiamulin and 8α-hydroxy-tiamulin accounts for 27% and 19%, respectively. The in vitro metabolism study showed that 40% of tiamulin was metabolized to mainly these three metabolites, which leads to the following quantitative estimations: 7% of tiamulin was hydroxylated in the 8α-position, 10% of tiamulin was hydroxylated in the 2β-position and finally 20% of tiamulin was deethylated.

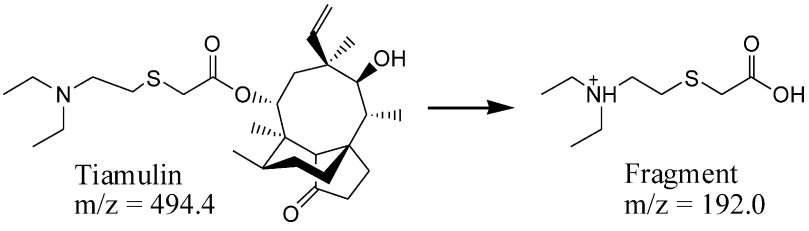
3.4. Identification of the metabolites

The tiamulin metabolites were analysed using tandem mass spectrometry to get some information about their structures. The fragmentation of tiamulin and the metabolites are shown in Table 2. It is seen that the fragmentation of the compounds is very simple, since the only fragment seen has the *m*/*z*-value 192.0, which corresponds to cleavage of the ester-bond. The same fragment is seen in the MS^2-spectra of metabolite M1 and M2, which proves that the hydroxylation had taken place in the mutilin-part (the ring system) of the molecules. Metabolite M3, M4 and M5 all give the same fragment with the *m*/*z*-value of 164.0, which is 28 less than the tiamulin-fragment (see Table 2). This is due to a loss of an ethyl-group at the nitrogen in the fragment. Metabolite M4 and M5 with the *m*/*z*-value 482 were both hydroxylated and deethylated. According to the fragmentation pattern the hydroxylations must have taken place in the mutilin-part of the molecules.

The ^{1}H NMR and ^{13}C NMR data of tiamulin and the isolated metabolites were determined from ^{1}H NMR, ^{13}C NMR, COSY, NOESY, DEPT, heteronuclear short range/one bond correlated (HMQC) and heteronuclear long range correlated (HMBC). ^{1}H NMR and ^{13}C NMR data of tiamulin and metabolite M1, M2 and M3 are compared in Tables 3 and 4, respectively. The numbering of tiamulin is shown in Fig. 1.

From the MS^2-data it was concluded that metabolite M1 is hydroxylated in the mutilin-part of the molecule. Comparing the ^{13}C NMR spectra and the ^{1}H NMR spectra of metabolite M1 and tiamulin, it is seen that the hydroxylation has taken place at either C-1 or C-2. The doublet at 4.31 ppm corresponds to the proton on the hydroxylated carbon, and in the NOESY spectrum there is a cross-peak between this proton and H-4. Therefore, the hydroxy-group must be placed in the β-position. One of the two protons in the carbon neighbour to the hydroxylated carbon has a cross-peak in the NOESY-spectrum to H-17, and the carbon must therefore be C-1. For that reason the hydroxy-group are placed

Table 2
Fragmentation of tiamulin and MS–MS data of tiamulin and the metabolites



Compound	Molecular ion $[M+H]^+$	Fragment (side chain)	$[M+H]^+$ – fragment (ring system)
Tiamulin	494.4	192.0	302.4
Metabolite M1	510.4	192.0	318.4
Metabolite M2	510.4	192.0	318.4
Metabolite M3	466.4	164.0	302.4
Metabolite M4	482.4	164.0	318.4
Metabolite M5	482.4	164.0	318.4

Table 3
^{1}H NMR data of tiamulin and metabolites

	Tiamulin	Metabolite M1	Metabolite M2	Metabolite M3
1	α: 1.65 (1H, m) β: 1.47 (1H, m)	α: 2.27 (1H, m) β: 1.19 (1H, d, *J* = 7.13)	1.73–1.83 (2H, m)	α: 1.69 (1H, m) β: 1.50 (1H, m)
2	α: 2.35 (1H, m) β: 2.19 (1H, m)	4.31 (1H, m)	α: 1.91 (1H, td, *J* = 15.5) β: 1.54 (1H, dt, *J* = 15.0)	α: 2.32 (1H, m) β: 2.24 (1H, m)
4	2.44 (1H, s)	2.85 (1H, s)	2.51 (1H, s)	2.54 (1H, s)
6	1.51 (1H, m)	1.80 (1H, m)	2.07 (1H, m)	1.54 (1H, m)
7	α: 1.38 (1H, m) β: 1.49 (1H, m)	1.45 (2H, m)	2.2–2.5 (2H, m)	α: 1.40 (1H, m) β: 1.56 (1H, m)
8	α: 1.10 (1H, m) β: 1.80 (1H, d, *J* = 14.58)	α: 1.04 (1H, dt, *J* = 4.8, 13.6) β: 1.92 (1H, dd, *J* = 13.6)	4.13 (2H, s)	α: 1.12 (1H, m) β: 1.86 (1H, m)
10	2.34 (1H, m)	2.34 (1H, p, *J* = 6.37)	2.2–2.5 (1H, m)	2.42 (1H, m)
11	3.54 (1H, d, *J* = 5.85)	3.58 (1H, d, *J* = 6.07)	3.62 (1H, d, *J* = 6.17)	3.60 (1H, d, *J* = 6.08)
13	α: 2.22 (1H, m) β: 1.33 (1H, m)	α: 2.23 (1H, m) β: 1.26 (dd, *J* = 14.96, 3.39)	α: 2.4 (1H, m) β: 1.37 (d, *J* = 16.25)	α: 2.24 (1H, m) β: 1.36 (1H, m)
14	5.65 (1H, d, *J* = 8.15)	5.59 (1H, d, *J* = 8.93)	5.65 (1H, d, *J* = 8.29)	5.72 (1H, d, *J* = 8.32)
15	1.39 (3H, s)	1.36 (3H, s)	1.46 (3H, s)	1.43 (3H, s)
16	0.68 (3H, d, *J* = 6.32)	0.71 (3H, d, *J* = 7.12)	0.72 (3H, d, *J* = 7.08)	0.71 (3H, d, *J* = 6.54)
17	0.90 (3H, d, *J* = 6.80)	0.92 (3H, d, *J* = 7.03)	0.93 (3H, d, *J* = 6.89)	0.94 (3H, d, *J* = 6.95)
18	1.12 (3H, s)	1.16 (3H, s)	1.16 (3H, s)	1.16 (3H, s)
19	6.31 (1H, dd, *J* = 17.38)	6.35 (1H, dd, *J* = 17.51)	6.32 (1H, dd, *J* = 17.59)	6.36 (1H, dd, *J* = 17.67)
20	Gem: 5.23 (1H, d, *J* = 12.14) Vic: 5.17 (1H, d, *J* = 17.48)	Gem: 5.21 (1H, dd, *J* = 11.28, 1.1) Vic: 5.28 (1H, dd, *J* = 17.53, 1.2)	Gem: 5.27 (1H, d, *J* = 11.10) Vic: 5.20 (1H, d, *J* = 17.55)	Gem: 5.21 (1H, d, *J* = 10.96) Vic: 5.17 (1H, d, *J* = 17.45)
22	A: 3.35 (1H, d, *J* = 15.60) B: 3.39 (1H, d, *J* = 15.72)[a,b]	A: 3.45 (1H, d, *J* = 15.98) B: 3.42 (1H, d, *J* = 15.89)[b]	A: 3.43 (1H, d, *J* = 16.07) B: 3.47 (1H, d, *J* = 15.89)[b]	A: 3.42 (1H, d, *J* = 15.99) B: 3.46 (1H, d, *J* = 16.06)[b]
23	2.94 (2H, t, *J* = 7.53)	2.96 (2H, t, *J* = 7.50)	2.30 (2H, t, *J* = 7.49)	2.95 (2H, t, *J* = 6.70)
24	3.35 (2H, t, *J* = 7.59)	3.38 (2H, t, *J* = 7.50)	3.40 (2H, t, *J* = 7.47)	3.29 (2H, t, *J* = 6.71)
25	3.23 (4H, q, *J* = 7.29)	3.25 (4H, q, *J* = 7.32)	3.26 (4H, q, *J* = 7.30)	3.15 (2H, q, *J* = 7.30)
26	1.29 (6H, t, *J* = 7.29)	1.33 (6H, t, *J* = 7.32)	1.32 (6H, t, *J* = 7.30)	1.34 (3H, t, *J* = 7.30)
NH				2.01 (s)

[a] The signals of H-22 and H-24 are overlaid, and the chemical shift values and the couplings are determined from the knowledge of the couplings of *N*-deethyl-tiamulin.
[b] It is a doublet of AB system.

at C-2. This is supported by the fact, that C-9 in metabolite M1 is shielded compared to C-9 in tiamulin, which correspond to a hydroxylation of the γ-carbon (C-2). The structure of metabolite M1 is therefore 2β-hydroxy-tiamulin.

The MS^2-data of metabolite M2 showed that also this metabolite is hydroxylated in the mutilin-part of the molecule. The ^{1}H NMR and ^{13}C NMR spectra indicate that the hydroxylation has taken place at C-7 or C-8, since the protons and the carbons at C-7, C-8 and C-9 are less shielded compared to tiamulin and C-6 is more shielded. The singlet with a chemical shift value of 4.13 in the ^{1}H NMR spectrum corresponds to the proton at the hydroxylated carbon. In the NOESY spectrum a cross-peak between this proton and H-17 is seen, and the proton must therefore correspond to H-8β. This is supported of a deshielding of C-9 in metabolite M2 compared to C-9 in tiamulin, which corresponds to a hydroxylation of the β-carbon (C-8). Moreover there is a cross-peak between H-8 and C-6 in the HMBC spectra, which also points to hydroxylation at C-8. Metabolite M2 is therefore 8α-hydroxy-tiamulin.

The ^{1}H NMR spectra of tiamulin and metabolite M3 are almost identical. H-24 and H-25 are a little more shielded in metabolite M3 than in tiamulin and the ^{1}H NMR spectra of metabolite M3 contains a singlet at 2.01 corresponding to a proton on the secondary amine. The integrations of H-25 and H-26 in the ^{1}H NMR spectra of tiamulin are two times the area of the integration of the corresponding protons in metabolite M3. The ^{13}C NMR spectrum of metabolite M3 has chemical shift values similar to the spectrum of tiamulin. The signals of C-25 and C-26 are much higher in the spectrum of tiamulin compared to the spectrum of M3, and the chemical shift values of C-24 and C-26 are a little more shielded and a little less shielded, respectively, in the spectrum of metabolite M3 compared to the spectrum of tiamulin. This suggests that one of the ethyl-groups at the amine is absent in the metabolite, and the structure must therefore be *N*-deethyl-tiamulin, which is in agreement with the MS^2 analysis of the compound.

Metabolite M4 and M5 were only formed in minor amounts and it was therefore not possible to isolate them in sufficient amounts to confirm their structures with NMR. MS and MS^2 analysis show, that they are deethylated in the site chain and hydroxylated in the mutilin-part. Both tiamulin and the impurity 11-oxo-tiamulin were deethylated by the microsomes. Therefore, it is most likely that the two hydroxylated metabolites were also deethylated. The deethylated metabolites were eluted few minutes before their parent compounds. The identity of the metabolites are therefore most likely M4: 2β-hydroxy-*N*-deethyl-tiamulin and M5: 8α-hydroxy-*N*-deethyl-tiamulin.

The structures of tiamulin and the metabolites are shown in Fig. 4.

Table 4
^{13}C NMR data of tiamulin and metabolites

	Tiamulin	Metabolite M1	Metabolite M2	Metabolite M3
1	26.40	36.94	25.58	27.19
2	36.58	73.18	36.56	37.53
3	173.26	[a]	[a]	188.44
4	60.08	57.84	57.30	61.10
5	43.64	43.68	43.78	44.52
6	38.79	38.03	32.80	39.65
7	28.63/28.55	29.23	39.21/39.34	29.38/29.22
8	32.06	35.47	74.41	32.81
9	47.52	38.03	53.13	47.35
10	37.75	38.41	39.21/39.34	38.58
11	76.38	77.73	77.78	77.45
12	45.71	46.75	46.75	45.78
13	46.59	47.29	47.45	46.64
14	73.08	74.49	74.12	74.34
15	16.52	17.01	16.81	17.26
16	18.29	18.85	18.54	18.88
17	12.93	12.93	13.76	13.32/13.60
18	28.63/28.55	29.23	29.42	29.38/29.22
19	141.85	142.85	142.92	143.00
20	118.18	119.11	119.09	119.00
21	172.40	[a]	173.95	174.02
22	35.94	36.94	36.82	36.70
23	28.01	28.97	28.96	30.92
24	52.41	53.37	53.38	48.36/48.45
25	49.52	50.46	50.48	48.36/48.45
26	10.17	11.03	11.04	13.32/13.60

[a] The signal was not observed due to a poor signal to noise ratio.

Earlier studies have shown that pleuromutilin derivates are often metabolized by hydroxylation in the 8α-, 1β- and 2β-positions [12]. In this work metabolites hydroxylated in the 8α- and 2β-positions were found and moreover metabolites deethylated at the amine were also found. These deethylated metabolites of tiamulin are in return mentioned in an environmental assessment report on the drug Denagard® containing tiamulin [13]. The report contains abstracts of some of the internal documents of the company Fermenta Animal Health Company. The following metabolism reactions are mentioned in the paper: *N*-dealkylation, monohydroxylations, epoxidations and conjugations.

According to the first summary report of tiamulin from EMEA [5] tiamulin is extensively metabolized to more than 15 metabolites in the livers of pigs. In this work we have only studied the NADPH dependent metabolism in pig liver microsomes, and we identified seven phase I metabolites. In addition several phase II metabolites would be expected to be formed in vivo.

NADPH is a cofactor of the Cytochrome P450 enzymes and flavin-containing monooxygenases (FMO) found in the microsomes. FMO primarily oxidizes nitrogen and sulphur in organic compounds. Such metabolites have not been found in this investigation of tiamulin, and the metabolism seen has therefore taken place in the Cytochrome P450 enzymes.

In the future it would be interesting to clarify the phase II metabolism in microsomes and to study the in vivo metabolism

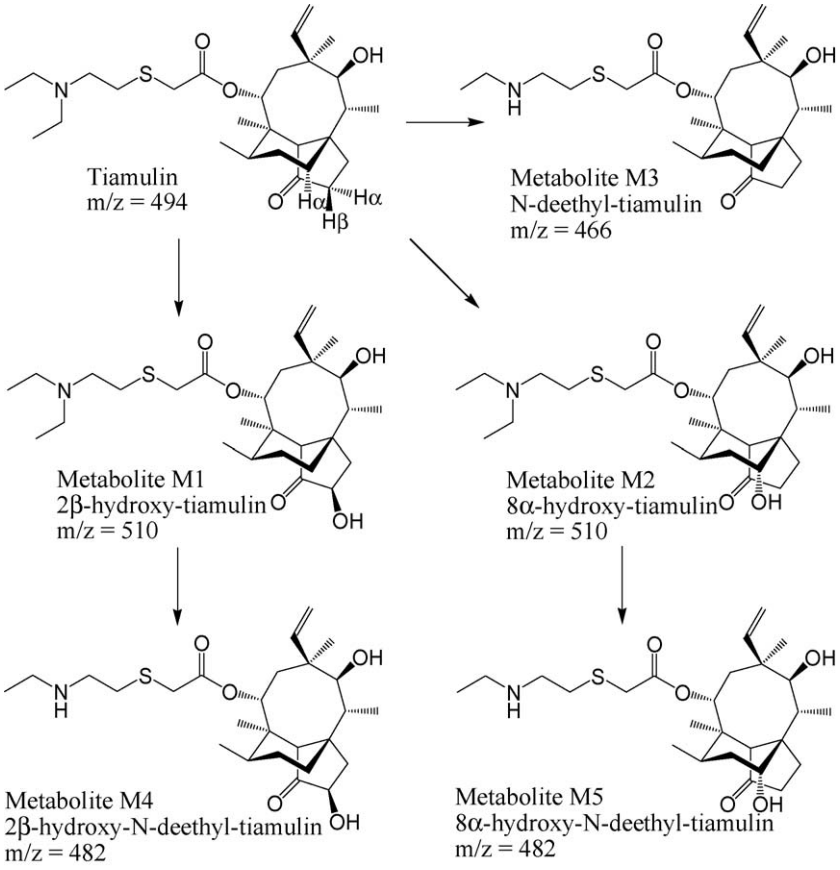


Fig. 4. Chemical structures of tiamulin and the five metabolites.

in pigs. To get a reliable quantification it would be necessary to use radiolabelled tiamulin.

4. Conclusion

Tiamulin was extensively metabolized by NADPH dependent enzymes in liver microsomes of pigs. Three metabolites were produced in large amounts, and they were isolated and structurally elucidated to be 8α-hydroxy-tiamulin, 2β-hydroxy-tiamulin and *N*-deethyl-tiamulin. Smaller amounts of two other metabolites were also produced and their structures were estimated to be 8α-hydroxy-*N*-deethyl-tiamulin and 2β-hydroxy-*N*-deethyl-tiamulin. The preparative purification had to be performed under alkaline conditions. The stability of the metabolites was lower under these alkaline conditions especially at room temperature, and precautions were made to avoid degradation of the metabolites isolated.

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