Research Article

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A mass spectrometric study on meloxicam metabolism in horses and the fungus *Cunninghamella elegans*, and the relevance of this microbial system as a model of drug metabolism in the horse

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This paper describes a study where the metabolism of the non-steroidal anti-inflammatory drug meloxicam was investigated in six horses and in the filamentous fungus *Cunninghamella elegans*. The metabolites identified were compared between the species, and then the fungus was used to produce larger amounts of the metabolites for future use as reference material. *C. elegans* proved to be a good model of phase I meloxicam metabolism in horses since all four metabolites found were the same in both species. Apart from the two main metabolites, 5′-hydroxymethylmeloxicam and 5′-carboxymeloxicam, a second isomer of hydroxymeloxicam and dihydroxylated meloxicam were detected for the first time in horse urine and the microbial incubations. Phase II metabolites were not discovered in the *C. elegans* samples but hydroxymeloxicam glucuronide was detected intact in horse urine for the first time in this study. Urine from six horses was further analyzed in a semi-quantitative sense and 5′-hydroxymethylmeloxicam gave peaks with much higher intensity compared to the parent drug and the other metabolites, and was detected for at least 14 days after the last given dose in some of the horses. From the results presented in this article, we suggest that analytical methods developed for the detection of meloxicam in horse urine after prohibited use should focus on the 5′-hydroxymethyl metabolite and that *C. elegans* can be used to produce large amounts of this metabolite for potential future use as a reference compound.

Keywords: meloxicam; metabolism; mass spectrometry; horse; *Cunninghamella elegans*

Introduction

Meloxicam [4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2*H*-1,2-benzothiazine-3-carboxamide-1,1-dioxide] is a non-steroidal anti-inflammatory drug (NSAID) that is cyclooxygenase-2 (COX-2) selective and belongs to the enolic acid group (Fig. 1). It is closely structurally related to other oxicams such as sudoxicam, piroxicam, isoxicam and tenoxicam[1]; although the methyl group on the thiazolyl ring has facilitated its metabolism and hence resulted in a shorter half-life compared to those of the others.[2] NSAIDs are extensively used in veterinary medicine due to their anti-inflammatory, analgesic and antipyretic properties.[3] Meloxicam is one of the newest drugs in this group and it is prohibited in the sports of horse racing.[4] For this reason it is important to develop powerful analytical methods for the detection of meloxicam in urine from race horses in the doping control laboratories. The finding of a prohibited substance in a sample could be either a finding of the substance itself or of a metabolite or isomer of the substance or the metabolite.[4] Some drugs are extensively metabolized in the body and it might be more efficient to search for a metabolite in the sample than for the parent compound. The metabolism of meloxicam in horses is only briefly discussed in the literature by Dumasia *et al*.[5] and de Kock *et al*.[6] The most commonly previously reported metabolites are formed by oxidation of the methyl group mentioned (Fig. 1), resulting in 5′-hydroxymethylmeloxicam and 5′-carboxymeloxicam. These two metabolites have been detected both *in vitro* and *in vivo* in different species such as humans,[7,8] thoroughbred horses,[5,6] rats,[2,8] mice and mini-pigs.[8] In earlier publications, the biotransformation of meloxicam has been investigated by the use of radioactivity monitoring after administration of ^{14}C-labelled meloxicam[2,8] and high performance liquid chromatography (HPLC) in combination with ultraviolet diode array detection (UV-DAD)[2] or either triple quadrupole mass spectrometry (MS)[8] or ion trap MS.[5,6]

Smith and Rosazza suggested that microorganisms could be used as models of metabolism of xenobiotic substances in mammals.[9] Since then, filamentous fungi such as *Cunninghamella elegans* have been used both as a complementary *in vitro* model for drug metabolism[10,11] and to produce

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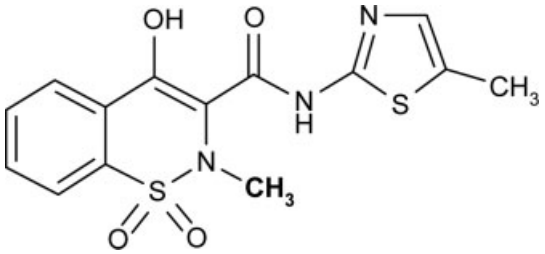

Figure 1. Chemical structure of meloxicam. The position of the three deuterium atoms of CD_3-meloxicam is on the *N*-methyl group in bold.

drug metabolites in amounts sufficient for complete structural confirmation[12,13] or further toxicological testing.[14,15] Some examples of drugs that have been metabolized by *C. elegans* are the antihistamines clemastine,[16] brompheniramine, chlorpheniramine and pheniramine[14]; the NSAIDs naproxen[17] and indomethacin[18]; antidepressants like amitriptyline[10] and mirtazapine[11]; and proton-pump inhibitors such as pantoprazole[19] and omeprazole.[20] Both phase I and phase II metabolism have been reported. Experiments with $^{18}O_2$ labelling and cytochrome P450 inhibitors have indicated that cytochrome P450 monooxygenase is expressed in *Cunninghamella*.[10] Activities of cytochrome P450 have been measured in microsomal preparations, activities of PAPS sulfotransferase, glutathione *S*-transferase and UDP-glucosyltransferase have been measured in the cytosol. Furthermore, UDP-glucuronyltransferase has been shown to be active in both microsomal and cytosolic preparations.[21] We wanted to examine if *C. elegans* is a useful model of metabolism of meloxicam in the horse, and if so, use the microorganism to produce larger amounts of the metabolites of interest for future use as reference compounds in the laboratory since drug metabolites often are not commercially available.

In summary, the aims of this study were: (1) to characterize the meloxicam metabolites excreted in urine from standardbred horses treated with meloxicam as well as the metabolites formed in *C. elegans* incubations and to compare the findings, (2) to use *C. elegans* for large-scale production of interesting metabolites for future use as reference compounds in the doping control laboratory, (3) to evaluate the relative concentrations of the drug and metabolites in urine from the horses and estimate the time after the last given dose of the parent drug and the main metabolites could be detected. The information gained will be useful in the development of new analytical methods for the detection of meloxicam in doping samples from race horses.

Experimental

Chemicals

Meloxicam sodium hydrate was purchased from Sigma Aldrich (Steinheim, Germany) and CD_3-meloxicam was obtained from Toronto Research Chemicals (ON, Canada). The water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA) and all chemicals and solvents used were of analytical grade or better and used without further purification. *Cunninghamella echinulata varians elegans*, American Type Culture Collection 9245, lot no 3656357 was purchased from LGC Promochem (Borås, Sweden). The Virkon-S, the saline solution, the Sabouraud agar plates, and the different kinds of broth needed for the experiment of *C. elegans* were obtained from the National Veterinary Institute (SVA) in Uppsala, Sweden.

Drug administration to the horses and urine sampling

Metacam® (meloxicam) was administered as an oral suspension to six healthy horses at Bjerke Dyrehospital in Oslo, Norway. The horses were standardbred trotters that weighed between 395 and 499 kg. The meloxicam dose given was 0.6 mg/kg bodyweight and was administered once daily for 5 days. A urine sample was collected 4 days prior to the first dose (the blank sample), on the third day of administration and every day thereafter for 21 days. The samples analyzed in this study were the ones taken day 5, 6, 8, 10, 13, 16 and 19. The urine samples were stored in −20 °C until analysis. The company that was responsible for the clinical trial also held the ethical permission for the study.

Microbial transformation of meloxicam

Cultures of *C. elegans* were grown on Sabouraud dextrose agar plates (mycological peptone 10 g/l, dextrose 40 g/l, agar 15 g/l) for 5 days in 27 °C and were then stored in 4 °C. The spores and mycelia from one agar plate were blended with 75 ml of sterile physiological saline solution in a sterile container. Five millilitres of the mycelia suspension were transferred to 250 ml Erlenmeyer flasks containing 30 ml of broth. Four different kinds of broth were compared at pH 5.5 and 7.0, those were yeast extract broth (yeast extract 8 g/l, sodium chloride 4.6 g/l, sucrose 20 g/l, monopotassium phosphate 3.75 g/l, disodiumphosphate 7.09 g/l), peptone broth (mycological peptone 8 g/l, sodium chloride 4.6 g/l, sucrose 20 g/l, monopotassium phosphate 3.75 g/l, disodiumphosphate 7.09 g/l), and two different Sabouraud dextrose broths (mycological peptone 10 g/l and dextrose 20 g/l or 40 g/l, respectively). The pH of the broth was adjusted from about 5.5 to 7.0 by the addition of 1.0 M phosphate buffer pH 6.9 (10–1000 ml of broth) and, when needed, 2.0 M sodium hydroxide. The different broths and pH were chosen with guidance of the extensive comparison of different cultivation media performed by Freitag *et al*.[22] The cultures were incubated for 3 days in 27 °C before the broth was decanted, and 30 ml of fresh broth was added along with meloxicam or a mixture of meloxicam and CD_3-meloxicam (100 µl, 7.0 mM in dimethylformamide). After 7 days in 27 °C the incubation was terminated by the addition of 25 ml of acetonitrile. The samples were stored at −20 °C until solid phase extraction. The Sabouraud dextrose broth with 20 g/l dextrose and pH 7.0 was used to prepare the cultures for meloxicam metabolite isolation.

Sample preparation

In vitro samples

The microbial samples were centrifuged (1000 *g* for 10 min) and the supernatants were removed and diluted with 45 ml of 1.0% formic acid in water. Varian abselut NEXUS solid phase extraction cartridges (200 mg, 6 ml, purchased from Scantec Lab AB in Partille, Sweden) were conditioned with 5 ml of methanol and 5 ml of Milli-Q water and equilibrated with 5 ml of 1.0% formic acid. The samples were applied and the cartridges were washed with 5 ml of 1.0% formic acid and left to dry under moderate vacuum for 5 min. The analytes were eluted with 5 ml of ethyl acetate. The eluate was evaporated to dryness under a stream of nitrogen gas at 60 °C.

In vivo samples

The pH of the horse urine samples (10.0 ml) was adjusted to 2.5 ± 0.3 with 1.0 M hydrochloric acid. Dichloromethane with 2%

isopropanol (20 ml) was added and the samples were mixed on a rotary mixer for 15 min followed by centrifugation at 1000 *g* for 15 min. The organic phase was transferred to a clean test tube and evaporated to dryness under a gentle stream of nitrogen at 50 °C. In the semi-quantitative study, 100 µl of CD_3-meloxicam (10 µg/ml) was added to the urine before the pH adjustment.

High performance liquid chromatography

HPLC-MS/MS experiments

Solvents in the mobile phase were 0.2% formic acid in water (A) and acetonitrile (B) and two different gradients were used. Gradient I: 20% B 0–5.0 min; 20–95% B 5.0–11.0 min; held at 95% B until 13.0 min; equilibration at 20% B 13.0–16 min. Gradient II: 20% B 0–2.0 min; 20–35% B 2.0–25.0 min; 35–90% B 25.0–26.0 min; held at 90% B until 40.0 min; equilibration at 20% B 40.0–50.0 min. The mobile phase was delivered at a flow rate of 200 µl/min by a quaternary Surveyor LC system (Thermo Electron Corp., San José, CA, USA) or a binary LC system of the 1100 series (Agilent Technologies, Waldbronn, Germany). The analytical column was a Phenomenex Luna 5 µ C_{18} (2), 150 × 2.00 mm, and the guard column was an ODS-C_{18} (4.0 × 2.0 mm, length × inner diameter) both from Scandinaviska Genetec AB (Västra Frölunda, Sweden).

Isolation of meloxicam metabolites

Three of the metabolites detected in this study were isolated from the *C. elegans* cultures by the use of a Waters Fraction Collector III (Waters Corp. Milford, MA, USA) in order to characterize them further by the use of MS^n experiments and nuclear magnetic resonance spectroscopy (NMR, data intended for a coming publication). The solvents in the mobile phase were the same as above and delivered as gradient III: 20% B 0–2.0 min; 20–35% B 2.0–25.0 min; 35–90% B 25.0–26.0 min; held at 90% B 26.1–32.0 min; equilibration at 20% B 32.1–40 min. The separation was achieved on a Hypersil™ GOLD column, 3 µm particles, 150 × 4.6 mm (Thermo Electron Corp., San José, CA, USA) and the volume injected was 50 µl. The flow rate was 1.0 ml/min and a split placed after the column directed at 15% of the flow to the mass spectrometer (a Finnigan LCQ ion trap) and the rest to the fraction collector. Three metabolites were collected in each run (suggested to be dihydroxymeloxicam 11 min 50 s–13 min 10 s, 5′-hydroxymethylmeloxicam 14 min 0 s–17 min 30 s, and 5′-carboxymeloxicam 20 min 0 s–23 min 0 s). The fractions collected from a total of 14 *C. elegans* cultures were pooled and freeze dried. For the MS^n experiments a small portion of each metabolite was dissolved in 0.2% formic acid in water and acetonitrile 50 : 50.

Mass spectrometry

Ion trap experiments

A Finnigan LCQ ion trap mass spectrometer (Thermo Electron Corp., San José, CA, USA) equipped with an electrospray interface was used in this study. Instrument control, data acquisition and data processing were carried out with the Xcalibur™ software, version 1.3. The electrospray ionization (ESI) and MS parameters were manually optimized for sensitivity during a 3 µl/min infusion of a meloxicam solution in methanol/water. The solution was mixed with the LC flow (0.2% formic acid in water/acetonitrile, 50 : 50, 200 µl/min) through a connecting T. In both positive and negative ion modes the capillary temperature was set to 225 °C, the spray voltage was 5 kV and the nitrogen sheath and auxiliary gas flow rates were 80 and 15 arbitrary units, respectively. The capillary voltage was set to 21 V (positive ESI) or −47 V (negative ESI). The ion trap was filled with helium gas and the MS^1, MS^2, MS^3 and MS^4 scan modes were used. The relative collision energy was optimized for each mass transition and ranged from 22% for MS^2 to 34% for MS^4 The wide band activation parameter was not used in these experiments.

Triple quadrupole experiments

A triple quadrupole TSQ Quantum Discovery instrument (Thermo Electron Corp., San José, CA, USA) with an electrospray ion source was used in the full scan modes of MS, MS/MS and neutral loss (NL) as well as selected reaction monitoring (SRM). ESI in the positive and negative mode was used and the sheath and auxiliary gas was nitrogen at 45–60 and 5–45 mTorr pressure, respectively. In the different experiments the capillary voltage was 3.5–4.8 kV and the temperature was 302–350 °C. For collision induced dissociation (CID) the collision cell was filled with argon at 1.5 mTorr and the collision energies used will be presented in the figure legends. Instrument control, data acquisition and data processing were carried out with the Xcalibur™ software, version 2.0 SR2. The mass transitions used in the SRM mode (positive ESI) were *m/z* 352 → 115 for meloxicam, *m/z* 355 → 115 for CD_3-meloxicam (the internal standard), *m/z* 368 → 131 for 5′-hydroxymethylmeloxicam, *m/z* 382 → 145 for 5′-carboxymeloxicam and *m/z* 384 → 131 for dihydroxymeloxicam. The collision energy was set to 20 V in the SRM mode.

Results and Discussion

Fragmentation of meloxicam

To interpret the mass spectra of the meloxicam metabolites it was necessary to first investigate the fragmentation of the parent compound. The CID of meloxicam was studied on an ion trap instrument during infusion of a meloxicam standard solution. In the positive ion mode (Fig. 2), a loss of water from protonated meloxicam ($[M + H]^+$ *m/z* 352) resulted in *m/z* 334, but the three major fragments in the MS^2 spectrum contained the thiazolyl function and were *m/z* 115 and 141, both previously reported,[5,6,23] and *m/z* 184. MS^3 of *m/z* 352 → 184 → scan showed that *m/z* 184 could be fragmented further into *m/z* 115, as well as the less abundant ions *m/z* 127, 141 and 156. See Fig. 2(c) for proposed structures of the fragments formed. The CID spectrum of CD_3-meloxicam, gave the same major fragments as meloxicam (*m/z* 115 and 141), as they did not contain the deuterated methyl group (results not shown). However, the fragment *m/z* 184 of meloxicam was shifted to *m/z* 187 for the isotopically labelled analog.

Figure 3 illustrates the MS^2, MS^3 and MS^4 spectra of meloxicam in the negative ion mode. The *m/z* for deprotonated meloxicam $[M - H]^-$ was 350 and the most intense peaks in the MS^2 spectrum were *m/z* 286 (loss of SO_2), *m/z* 210 (deprotonated benzothiazine) and *m/z* 146. This fragmentation has been proposed earlier by de Kock *et al.*[6] Further, MS^3 of 350 → 286 → scan (Fig. 3(b)) as well as 350 → 210 → scan (spectrum not shown) proved that the *m/z* 146 fragment could be formed from either *m/z* 286 or 210. The *m/z* 252 fragment was formed from *m/z* 286 most probably by the loss of H_2S (34 Da). In the MS^4 spectrum *m/z* 350 → 286 → 146 → scan, *m/z* 128 (loss of water) and *m/z*

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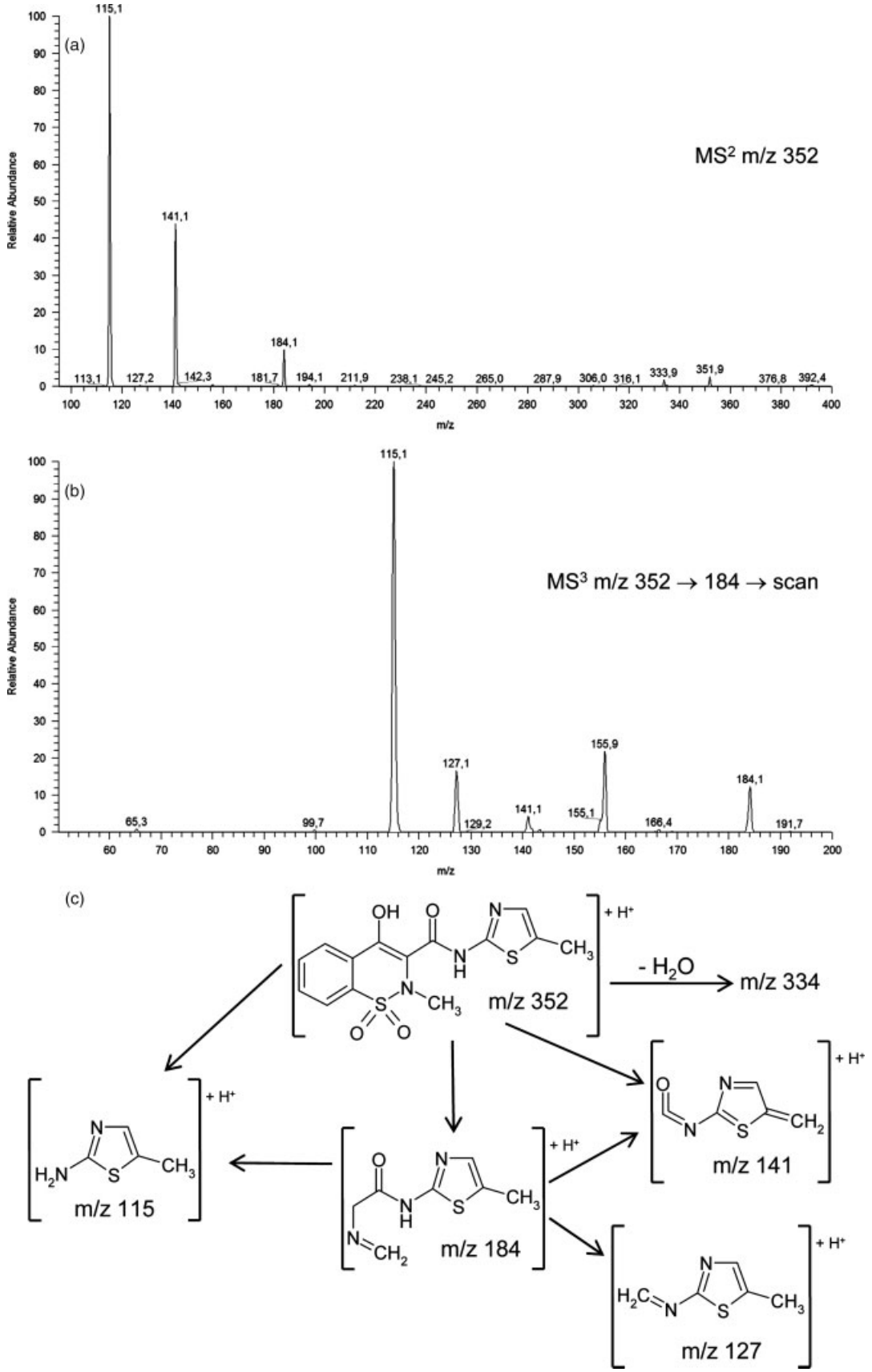

Figure 2. Positive ESI MS^n analysis of meloxicam standard. Data was collected for 60 s during a constant infusion of the solution into the ion trap instrument. (a) MS^2 spectrum of *m/z* 352, the collision energy was 22%. (b) MS^3 spectrum of *m/z* 352 → 184 → scan, the collision energies were 22 and 25%, respectively. For details, see section on Experimental. (c) Proposed structures of the fragments.

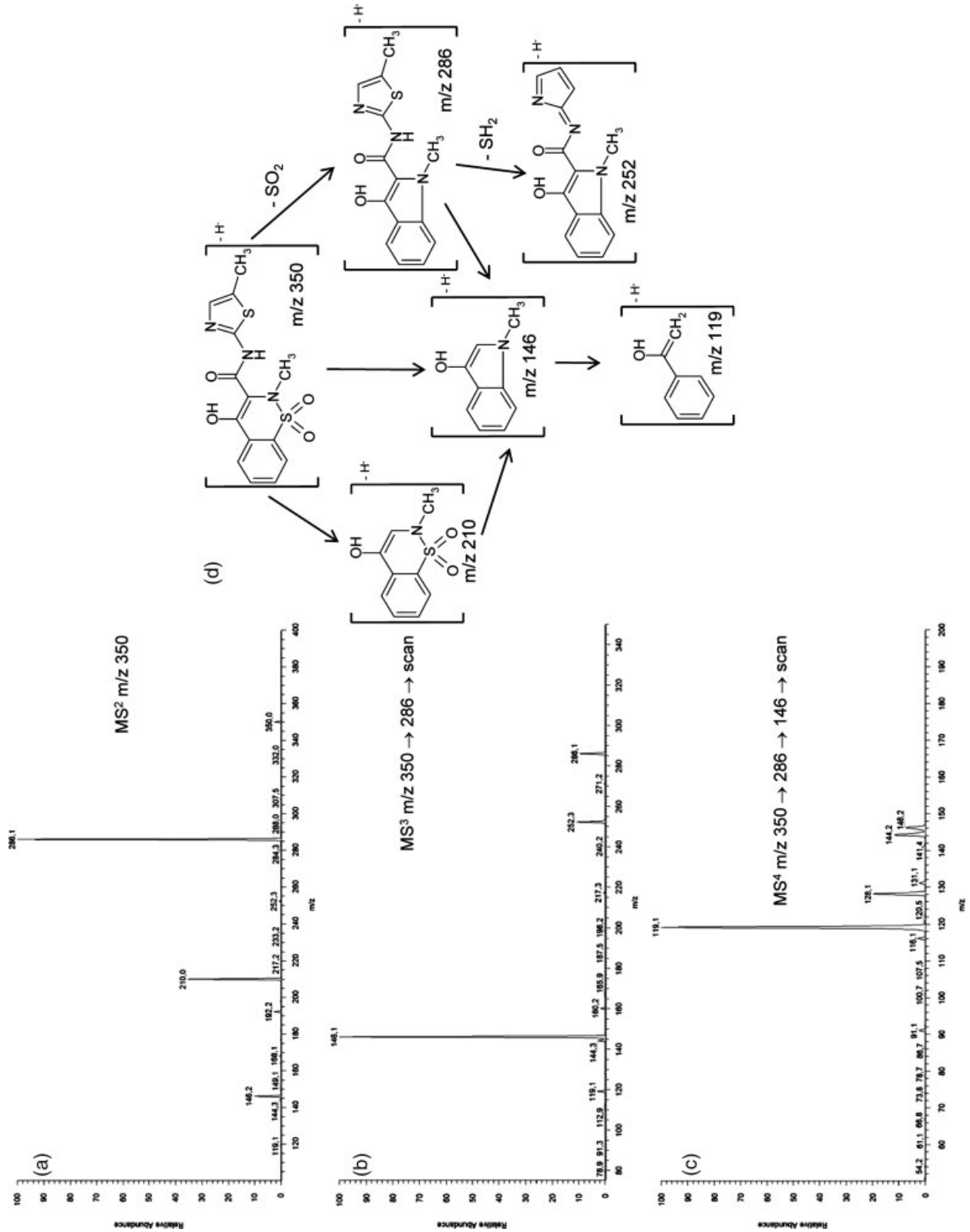

Figure 3. MS^n analysis of meloxicam in the negative ion mode on the ion trap instrument. The meloxicam standard solution was injected as a constant infusion and data was collected for 60 s. (a) MS^2 spectrum of *m/z* 350, the collision energy was 26%. (b) MS^3 spectrum of *m/z* 350 → 286 → scan, the collision energies were 26 and 25%, respectively. (c) MS^4 spectrum of *m/z* 350 → 286 → 146 → scan, the collision energies were 26, 25 and 34%, respectively. For details, see section on Experimental. (d) Proposed structures of the fragments.

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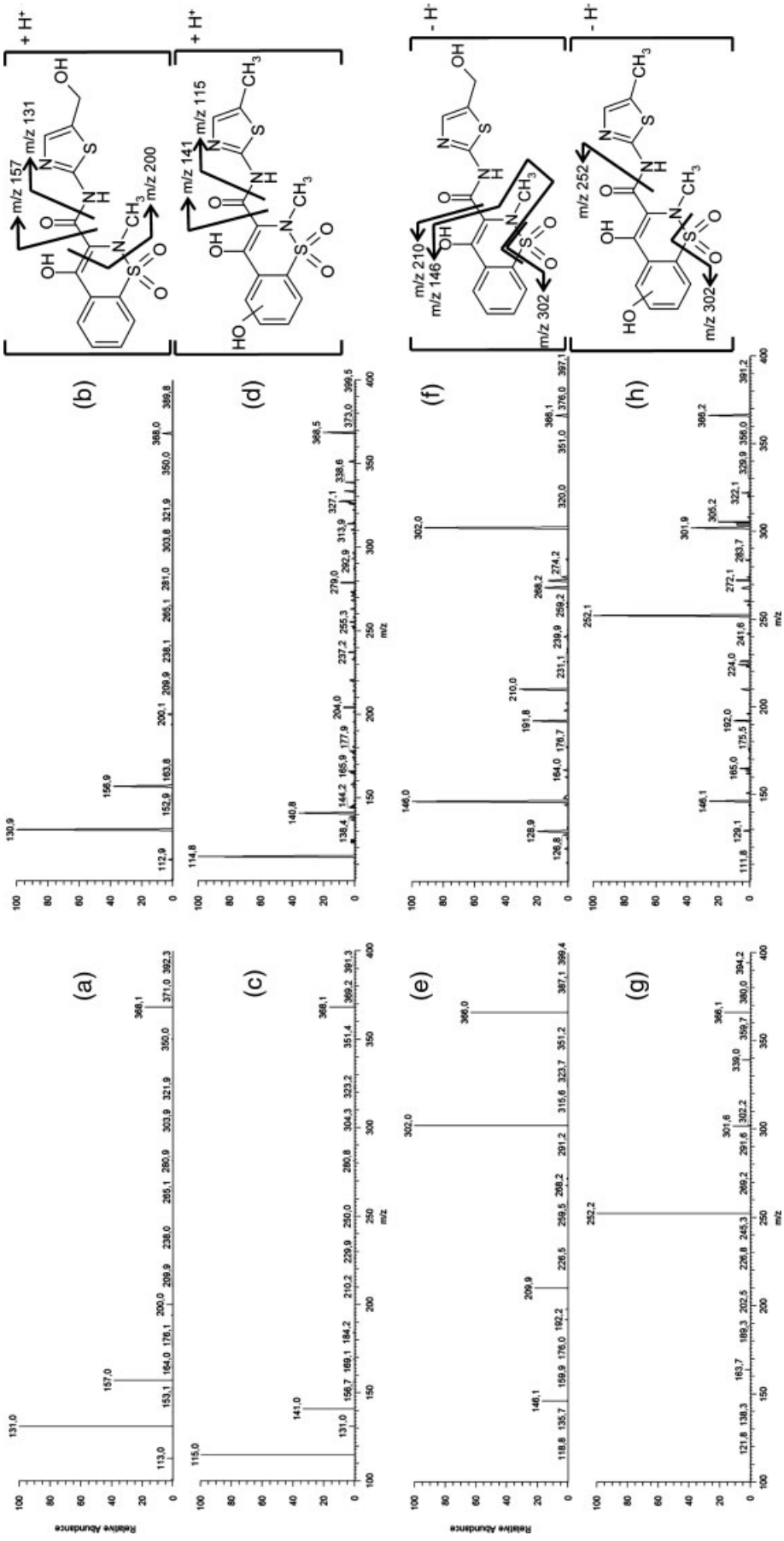

Figure 4. The spectra to the left derive from a *C. elegans* sample (gradient I used for the HPLC) and the ones on the right from a horse urine sample (HPLC gradient II used). (a) and (b) illustrate spectra from the most intense peak after positive ESI MS/MS of *m/z* 368, proposed to correspond to 5′-hydroxymethylmeloxicam, and (c) and (d) are spectra from the less intense peak in the chromatograms of the same samples suggested to correspond a second isomer of hydroxylated meloxicam. In the negative ion mode, MS/MS of *m/z* 366 also resulted in two peaks which spectra are presented in (e) and (f) from the major peak, and (g) and (h) from the minor peak. The instrument used was a triple quadrupole and the collision energy was 15 V in (e) and (g) and 20 V in the others. For more information, see section on Experimental.

119 were the most abundant ions. Structural suggestions of the major fragments are presented in Fig. 3(d). For CD_3-meloxicam, the major fragments in the CID spectrum contained the deuterated methyl group, resulting in a shift of 3 *m/z* units compared to meloxicam, giving the peaks *m/z* 149, 213 and 289 (results not shown).

Meloxicam metabolites in C. elegans incubations and horse urine

A way to facilitate the identification of drug metabolites in complex matrices is to administer a mixture of the drug and its deuterium labelled analog and then look for the resulting doublet peaks in the full scan mass spectra.[24] The presence of the mass shift between the fragment ions of the drug and the deuterium labelled drug can also make the interpretation of noisy mass spectra easier. We applied this approach in our search for phase I metabolites produced by *C. elegans* and added a one-to-one mixture of meloxicam and CD_3-meloxicam to the microbial incubations. The result was a shift of three in the *m/z* of the molecular ions and their fragment ions for the biodegradation products formed from meloxicam and deuterium labelled meloxicam. The result was a faster sifting of ions of interest from the many spectra produced.

Four kinds of broth were compared at pH 5.5 and 7.0 in order to find the optimum cultivation medium for meloxicam metabolite production. Approximately 75–90% of the meloxicam added to the *C. elegans* cultures was biotransformed into several metabolites. The different kinds of broths did not result in any major differences in meloxicam metabolite formation except for the yeast broth at pH 7.0 that resulted in significantly lower yield of metabolites. The Sabouraud dextrose broth (mycological peptone 10 g/l and dextrose 20 g/l, pH 7.0) was chosen for the metabolite isolation cultures.

The *C. elegans* samples were cleaned up and concentrated by solid phase extraction, and the urine samples by liquid–liquid extraction, before analysis with the triple quadruple mass spectrometer (for details see section on Experimental). No significant differences were observed between the MS/MS spectra of meloxicam produced with the triple quadrupole compared to the ion trap instrument, in neither the positive nor the negative ion mode and thus, the identification of the metabolites was achieved by the interpretation of the mass spectral data from the triple quadrupole mass spectrometer and comparison with the fragmentation of the meloxicam standard solution from the ion trap instrument. The results are summarized below.

Two isomers of hydroxymeloxicam

One of the main metabolites reported in both humans[7] and horses[5,6] is 5′-hydroxymethylmeloxicam ($[M + H]^+$ *m/z* 368 and $[M - H]^-$ *m/z* 366). HPLC-MS/MS analysis in the positive mode of *m/z* 368 of the *C. elegans* and horse urine samples, gave two peaks in the total ion chromatogram (TIC) that were suspected to represent hydroxylated metabolites (not shown). The spectra from the major peak (*C. elegans* Fig. 4(a) and horse urine 4(b)) were well in agreement with those of the 5′-hydroxymethylmeloxicam metabolite, previously described in the literature.[5,6] The most abundant fragments in the spectrum of the minor peak were *m/z* 115 and *m/z* 141 (*C. elegans* Fig. 4(c) and horse urine 4(d) *cf.* 2(a)) were the same as for meloxicam, indicating a second isomer of oxidized meloxicam where the oxidation was not on the thiazolyl moiety.

The negative ion mode MS/MS spectrum of the major peak is shown in Fig. 4(e) and (f), fungus and horse urine respectively. The spectra had peaks at the same *m/z* values as the previously published ones for 5′-hydroxymethylmeloxicam.[6]

The MS/MS spectrum for the minor peak in the negative ion mode is shown in Fig. 4(g) (fungus) and (h) (horse urine). The *m/z* 302 fragment probably corresponded to meloxicam +16 $-SO_2$, but the most abundant fragment was *m/z* 252 (*m/z* 255 for the deuterium labelled metabolite) which may be derived from the cleavage of the amide bond. The hydroxylation has most likely occurred on the benzothiazine moiety, but the exact position could not be concluded. Similar metabolites of piroxicam have been discovered in rat urine but the sites of the hydroxylation for the two isomers were not determined closer than in position 5, 6 or 7 on the benzothiazine ring.[25] An *m/z* 252 fragment was found in meloxicam standard as well (Fig. 3, suggested to be derived from meloxicam $-SO_2$ $-H_2S$) and most likely these are two completely different fragments although they have the same nominal *m/z* value. However, a fragment corresponding to the cleavage of the amide bond (*m/z* 236) could not be found in the meloxicam standard. The reason for this might be that the aromatic oxidation in the metabolite shifted the localization of the deprotonation and hence affected the fragmentation. This is one example of a case where it was useful to be able to compare the spectra with those of the deuterium labelled meloxicam to make sure that the *m/z* 252 fragment was really derived from the meloxicam metabolite although the two *m/z* 252 fragments could not be identical.

5′-Carboxymeloxicam

Another main metabolite of meloxicam that has been previously reported in humans, rats, mice, mini-pigs[8] and horses[5,6] is 5′-carboxymeloxicam, formed by further oxidation of 5′-hydroxymethylmeloxicam. In Fig. 5 the positive and negative electrospray MS/MS spectra of the peaks corresponding to $[M + H]^+$ *m/z* 382 and $[M - H]^-$ *m/z* 380, respectively, from a *C. elegans* sample are presented. The positive electrospray spectra from *C. elegans* (Fig. 5(a)) and the horse urine samples (results not shown) were well in agreement with the one published by Dumasia *et al.*, demonstrating that *C. elegans* can metabolize meloxicam into 5′-carboxymeloxicam.[5] Also in the negative ion mode, the spectra were in agreement with that found in the literature (Fig. 5(b) *cf.* ref. [6]).

Hence, meloxicam could be metabolized to 5′-carboxymeloxicam in both horses and *C. elegans*. Even though we identified both 5′-hydroxymethylmeloxicam and 5′-carboxymeloxicam in both species we did not detect any intermediate aldehyde metabolite.

Dihydroxymeloxicam

C. elegans is known to oxidize xenobiotic substances extensively[10,11] and therefore we were encouraged to look for other oxidized metabolites as well. Interestingly, MS/MS of *m/z* 384 ($[M + H]^+$ for meloxicam + 32) resulted in a peak in the TIC for both the *C. elegans* sample and the horse urine sample. This metabolite has never been reported before and we used a fraction collector to isolate it from the *C. elegans* cultures, together with the two main metabolites, in order to confirm the structures by the use of NMR (a future study) and maybe be able to use the metabolites as reference material in the doping control laboratory. The isolated metabolites were also analyzed in the MS^2 and MS^3

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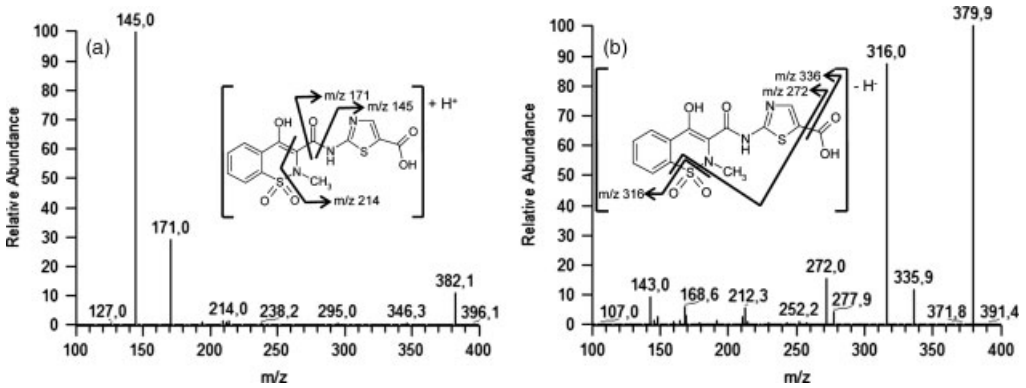

Figure 5. Triple quadrupol MS/MS spectra and fragmentation suggestions of 5′-carboxymeloxicam from a *C. elegans* sample in positive (a) and negative (b) ESI. The precursor ions were *m/z* 382 and 380, respectively, the collision energy was 15 V, and HPLC gradient I was used. For further information, see section on Experimental.

modes of the ion trap mass spectrometer, and the MS^2 spectra of the suspected dihydroxylated metabolite in positive and negative ESI are illustrated in Fig. 6(a) and (b), respectively. The ions *m/z* 131, 157 and 200 (Fig. 6(a)) were the same as those found from 5′-hydroxymethylmeloxicam (*cf.* Figure 4(a)) and the other oxidation must consequently be on the benzothiazine moiety. In Fig. 6(b) the main fragments were *m/z* 162, *m/z* 226, *m/z* 252, and *m/z* 318 (302 + 16 *m/z* units, *cf.* Figure 4(g)). When the horse urine samples were analyzed on the triple quadrupole instrument the most abundant ions in the spectra were *m/z* 131 and 157 in positive ESI (Fig. 6(c)) and *m/z* 252 and 318 in negative ionization (Fig. 6(d)). Thus, it appears to be possible for the oxidative enzymes in *C. elegans* and in the horse to either perform oxidation in one of the two positions in meloxicam or in both. We find it interesting that the fungus *C. elegans* appeared to metabolize this drug in the same way as horses, a fact that can be useful in future studies on drug metabolism in horses.

Other phase I metabolites

According to the literature, NSAIDs of the oxicam family can be biotransformed to metabolites where the amide bond has been broken, e.g. a carboxybenzothiazine and a benzothiazine metabolite of piroxicam have been detected in rat, dog, monkey and man.[1] Another metabolite that is considered typical for oxicams is the oxoacetic acid metabolite.[1,7] However, in this study none of these metabolites was detected.

Hydroxymeloxicam glucuronide

In 2002, Dumasia *et al*. concluded that some increase in the concentration of the two main metabolites of meloxicam (i.e. 5′-hydroxymethylmeloxicam and 5′-carboxymeloxicam) could be observed after enzymatic and base hydrolysis.[5] In another publication, de Kock *et al*. could not observe any difference in the yield of meloxicam or its metabolites after treatment of the samples with arylsulfatase and/or β-glucuronidase.[6] *O*-glucuronides have been detected in bile from humans and rats after oxidation of the thienothiazine moity of tenoxicam resulting in 7- or 8-*O*-glucuronyltenoxicam and in human urine after oxidation of the benzothiazine group in piroxicam yielding 5′-*O*-glucuronylpiroxicam.[1]

In our study, the horse urine was diluted and injected without further sample preparation into the triple quadrupole mass spectrometer in order to search for phase II metabolites. MS/MS scans of *m/z* for possible conjugates were applied and in Fig. 7 the TIC, extracted ion chromatograms for *m/z* 544 and 368, and the spectrum from the peak at 28.3 min (gradient II) after MS/MS of *m/z* 544 in positive ESI are presented. Hydroxymeloxicam glucuronide would result in $[M + H]^+$ *m/z* 544, and as can be seen in the spectrum the *m/z* 544 ion disintegrated into *m/z* 368 (which corresponds to hydroxymeloxicam) upon CID. Thus, these data clearly indicates that a glucuronic acid conjugate of meloxicam can be formed in the horse. Neutral loss scans of 80 and 176 Da (for sulfate and glucuronic acid metabolites, respectively) did not result in any additional findings in the horse urine samples, and no conjugates could be detected in the *C. elegans* samples that had been extracted with SPE.

Meloxicam and its metabolites in urine from healthy horses

Nine healthy horses were a part of a pharmacokinetic study of orally administered meloxicam at the Bjerke Veterinary Clinic in Norway. The horses were divided in groups where one served as control (three horses) and the remaining six were treated with meloxicam. In this study we were interested in comparing the levels of meloxicam (M) and its main metabolites 5′-hydroxymethylmeloxicam (M1), 5′-carboxymeloxicam (M2) and dihydroxymeloxicam (M3) in urine from the six horses, as well as investigating for how long time after the treatment was ended the substances could be traceable in the urine samples. The only substances available as synthetic standards were meloxicam and CD_3-meloxicam why a proper quantitative analysis of the metabolites was not possible. Instead we made a relative estimate based on the ratios between the area of the SRM-peak of the substance M, M1, M2 or M3 and the SRM-peak of the internal standard (CD_3-meloxicam). This approach worked well for the purpose of this study.

The urine samples were cleaned up and concentrated by liquid–liquid extraction before they were injected into the LC-MS/MS system. The retention times for the substances with gradient II were 29.7 min (M and CD_3-meloxicam), 28.4 min (M1), 30.8 min (M2) and 25.5 min (M3), see Fig. 8.

The six horses in this study showed great similarities in their meloxicam metabolism and excretion patterns. In Fig. 9 the peak area ratio was plotted against time for a representative horse in the study. The maximum concentration of the parent drug and the two main metabolites 5′-hydroxymethylmeloxicam and 5′-carboxymeloxicam in horse urine seemed to be reached on

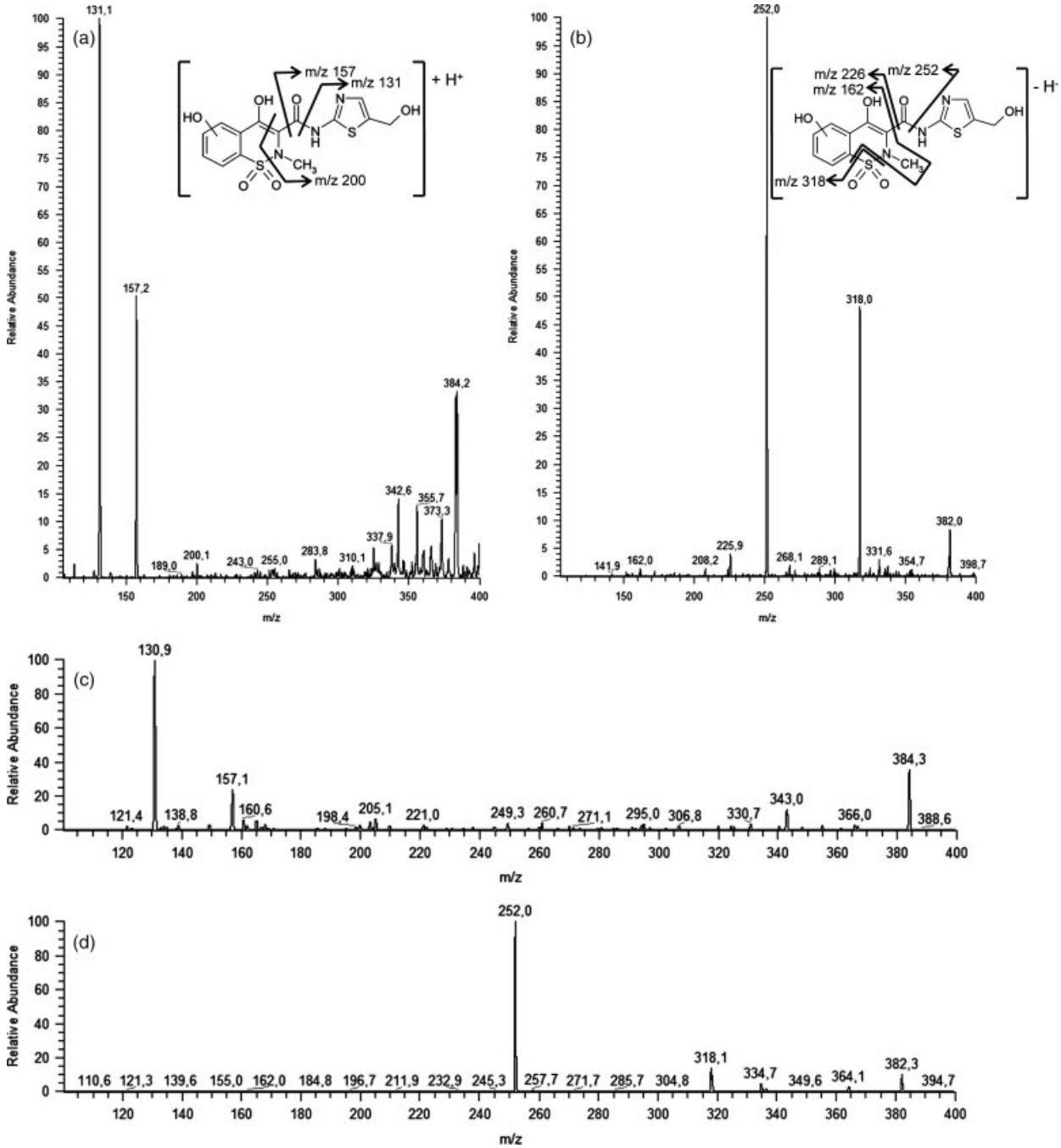

Figure 6. MS^2 spectra and fragmentation suggestions of dihydroxymeloxicam isolated from *C. elegans* cultures in positive (a) and negative (b) ESI. The precursor ions were *m/z* 384 and 382, respectively. The isolated metabolite was injected into the ion trap instrument as a constant infusion (8 µl/min). Spectra from MS/MS of $[M + H]^+$ *m/z* 384 (c) and $[M - H]^-$ *m/z* 382 (d) of a horse urine sample in the triple quadrupole instrument (HPLC gradient II). For more information, see section on Experimental.

day 5 or 6, i.e. the last day of treatment or the day thereafter. Interestingly, the intensity of the 5′-hydroxymethylmeloxicam peak is about three times higher than that of the parent drug meloxicam, a fact that could be useful in the development of new analytical methods for the detection of this drug, e.g. for doping control purposes. Toutain *et al*. report that the meloxicam concentration in horse urine was below the limit of quantification for the analytical method within 3 days after the final dose.[26] In our study the main part of both the metabolites and meloxicam seem to be excreted 3 days after the treatment is ended, however the 5′-hydroxymethyl metabolite was detectable for at least 14 days after the last administration of the drug in some of the horses.

There were two differences in our findings compared to the Dumasia and de Kock publications. Firstly, the main metabolite in urine from all six horses in this study was 5′-hydroxymethylmeloxicam, not 5′-carboxymeloxicam as in the refs. [5,6]. Secondly, unchanged meloxicam was not the major component found in urine from the horses in our study, 5′-hydroxymethylmeloxicam was. These differences might be

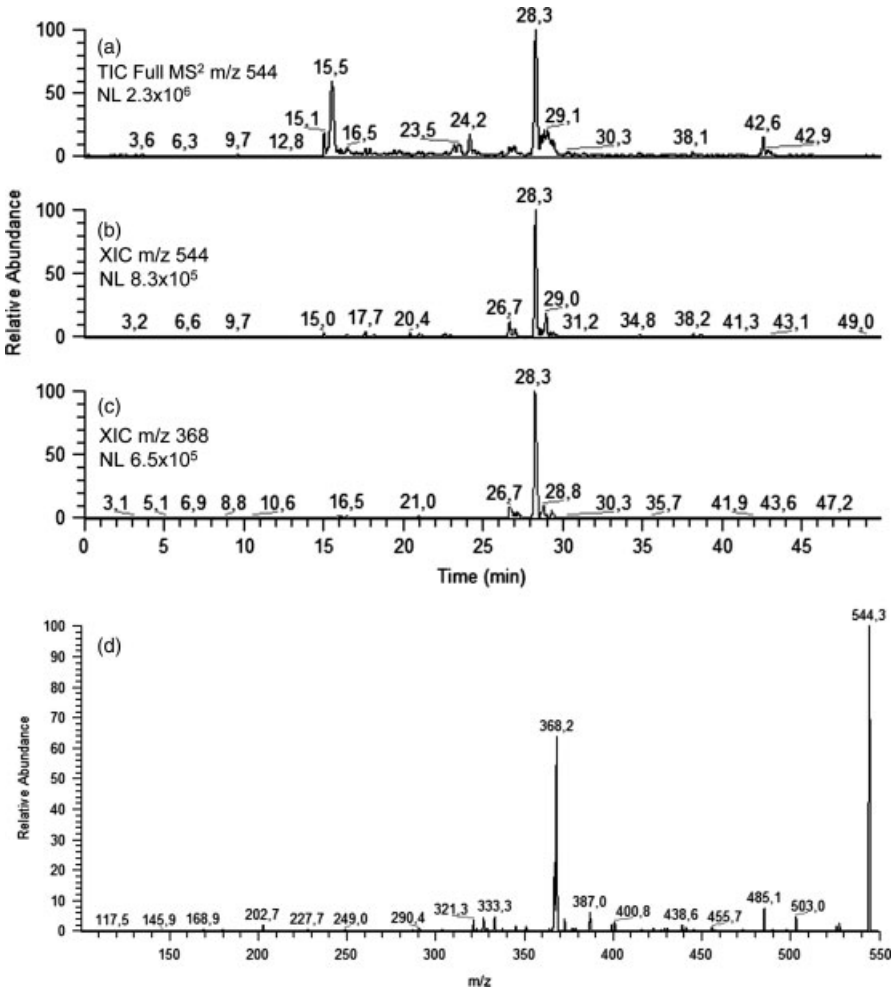

Figure 7. Chromatograms of MS/MS of *m/z* 544 of a horse urine sample (a–c) and mass spectrum of the peak at 28.3 min (d). Gradient II was used, the instrument was the triple quadrupole, the ionization was positive ESI, and the collision energy was 20 V. For other details, see section on Experimental.

explained by the use of different races since the horses in our study were standardbred trotters while Dumasia and de Kock used thoroughbred race horses.

Conclusions

Four phase I metabolites of meloxicam were detected in horse urine and *C. elegans* cultures in this study. A second isomer of hydroxylated meloxicam and the dihydroxylated metabolite are described for the first time in this publication together with the two main metabolites, 5′-hydroxymethylmeloxicam and 5′-carboxymeloxicam, that have been previously reported in horse urine. To our knowledge, the metabolism of meloxicam has not been studied in the fungus *C. elegans* before and we found it interesting that the metabolites detected were the same in both species. Thus, the fungus proved to be a good model of the phase I metabolism of meloxicam in horses and it was also illustrated that *C. elegans* can be used to produce larger quantities of the metabolites in a cheap and simple manner. Concerning phase II metabolism, an intact glucuronic acid conjugate of hydroxymeloxicam was detected in horse urine for the first time in this study, but conjugates were not detected in the microbial samples.

Additionally, a semi-quantitative analysis of meloxicam, 5′-hydroxymethylmeloxicam, 5′-carboxymeloxicam and dihydroxymeloxicam was conducted with urine from six standardbred trotters that had received meloxicam orally for 5 days. Interestingly, 5′-hydroxymethylmeloxicam resulted in SRM peaks with three times larger area than that of the parent drug and also proved to be detectable in urine from some of the horses for at least 14 days after the treatment ended with the analytical method used.

Adding up the results presented in this article, it seems advantageous to choose the 5′-hydroxymethyl metabolite for detection instead of meloxicam itself in the development of analytical methods in the anti-doping programmes in horse racing. Furthermore, the fungus *C. elegans* can be used to produce drug metabolites for reference compound usage and appears to be a promising model for phase I drug metabolism in horses.

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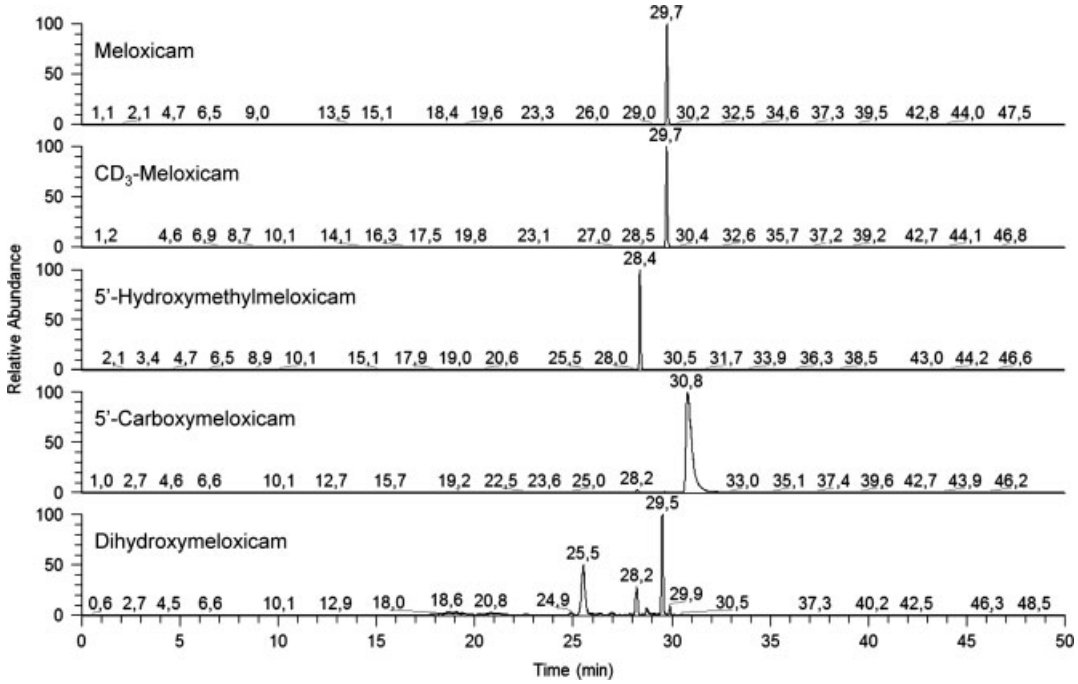

Figure 8. SRM chromatograms for (from top to bottom) meloxicam, CD_3-meloxicam (the internal standard), 5′-hydroxymethylmeloxicam, 5′-carboxymeloxicam and dihydroxymeloxicam from a urine sample from one of the horses in the study. The HPLC gradient II was used, for details, see section on Experimental.

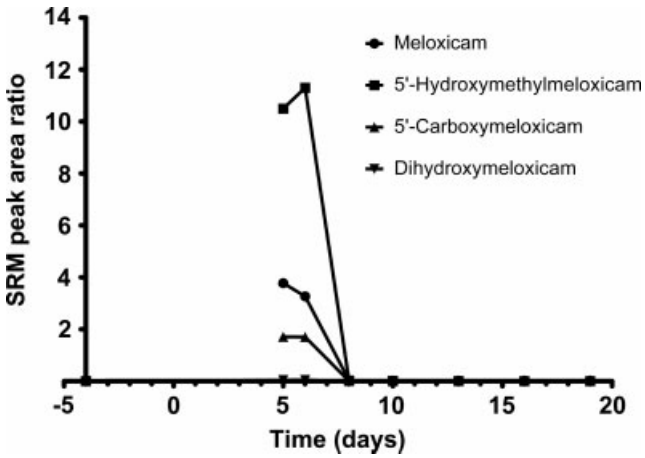

Figure 9. A plot with SRM-peak area ratio *versus* time for meloxicam, 5′-hydroxymethylmeloxicam, 5′-carboxymeloxicam and dihydroxymeolxicam for one of the horses in the study. The blank sample was collected 4 days prior to the first dose, the first dose is administered at day 1 in the graph and the first sample analyzed is the one taken on the fifth day of administration.

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