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Characterization of *in vivo* plasma metabolites of tepoxalin in horses using LC–MS–MS

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ABSTRACT

Tepoxalin is a veterinary drug registered for use in the dog as a dual inhibitor (cyclooxygenase - 5 lipoxygenase). In the horse, it predominantly triggers a strong cyclooxygenase inhibition; this bias seems to be due to the action of its metabolite(s). Among these, only the RWJ-20142 is well known, while to the best of our knowledge no information is available on the other metabolites produced *in vivo*. Hence, the identification of its main metabolic pathway is pivotal to better understand its clinical activity.

A suitable high performance liquid chromatography method has been applied to liquid chromatography–mass spectrometry for the characterization of the main metabolites in plasma of horses orally treated with tepoxalin. Mass spectrometry in full scan, product ion scan and precursor ion scan modes, provided information useful in elucidating large parts of the structure of the unknown metabolites detected. These structures are closely related to that of tepoxalin. One of these metabolites was speculated to be a structural isomer of the parental drug.

These findings could be important to understand the pharmacology of tepoxalin in horses.

1. Introduction

Chronic inflammatory diseases, such as osteoarthritis, are one of the most important causes of reduced performance in animals, especially in horses [1]. Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used therapy [2]. Tepoxalin is a dual inhibitor NSAID with analgesic, anti-inflammatory, and antipyretic properties. It has recently been registered as a veterinary drug. Because of the inhibition of 5 lipoxygenase (5-LO), tepoxalin may offer a novel approach both to the preservation of gastro-intestinal mucosal integrity and reduction of bronchoconstriction [3]. Tepoxalin has been approved for use in dogs by the European Committee for Medicinal Products for Veterinary Use as Zubrin (Schering-Plough) [4].

Studies conducted in rats [5], chickens [6], rabbits [7], dogs [5,8] horses [9] and humans [10], reported maximum plasma concentrations were reached between 0.7 and 4 h after oral tepoxalin administration. Tepoxalin is also rapidly converted to its active metabolite RWJ-20142. The latter molecule is produced in large amounts, has a long plasma half-life and seems to possess cyclooxygenase-1 (COX-1) inhibitor activity only. In chickens and horses, the presence of a new unknown metabolite has been speculated based on the chromatographic data [6,11]. The characterization of metabolic compounds, used in research and clinical programs, is an essential prerequisite for use of a drug or its registration in a new animal species. Species differences in drug metabolic fate are, in most cases, the primary source of variation in drug disposition, and, therefore in drug activity and toxicity across species. An important element of the clinical characterization of the drug effect is the identification of metabolic products and their activities. The number and quantity of these compounds can vary from species to species and they are often present in very low concentrations (ca. 0.01–0.5%). This does not mean that these products can be ignored because they could exhibit a large range of activities; from high toxicity up to high effectiveness. Separation of such *in vivo* formed compounds, for detailed structure elucidation, is the first step of this process. An on-line technique capable of providing structural information can be of great value. Through the use of capabilities that allow direct interfacing to HPLC, mass spectrometry can rapidly provide data to assist in the identification and characterization of drug metabolite products. The aim of the present study is to identify the metabolic pathway of tepoxalin in the plasma of horses 10 h following oral administration.

Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; 5-LO, 5 lipoxygenase; COX-1, cyclo-oxygenase-1; DP, declustering potential; IS, ionspray voltage; CUR, curtain gas; GS1, gas source 1; GS2, gas source 2; TEM, source temperature; EP, entrance potential; MS2, tandem mass spectrometry; EP, entrance potential; CAD, nitrogen as a collision; CE, collision energy.

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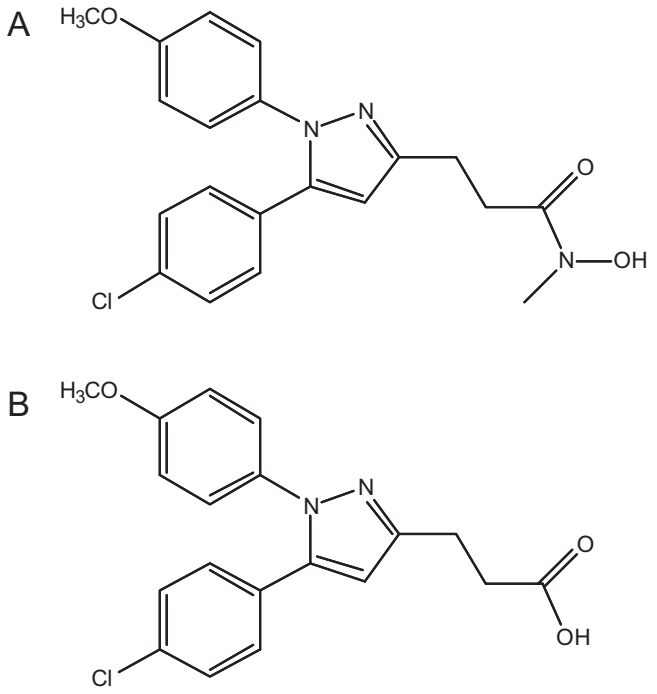

Fig. 1. Molecular structure of (A) tepoxalin, (B) its active metabolite (RWJ-20142).

2. Materials and methods

2.1. Chemicals

Tepoxalin 5-(4-chlorophenyl)N-hydroxy-1(4-methoxyphenyl)-N-methyl-1H-pyrazole-3-propamide) (99.9% pure) and its acid metabolite (RWJ-20142) (3-[5-(4-chlorophenyl)-1-(4-methoxyphenyl)-1H-pyrazol-3-yl]-propanoic acid) (99.7% pure) (Fig. 1) standard powders, were a gift from Schering-Plough Co. (Summit, NJ, USA). HPLC-grade water from Baker (Baker Analyzed® Reagent, J.T. Baker, Deventer, Holland) was used for buffer preparation. The methanol and acetonitrile used were of HPLC grade from Carlo Erba (Milan, Italy). Acetic acid, methyl-t-butyl ether, sodium phosphate and ammonium acetate, were of analytical-reagent grade from Sigma (St. Louis, MI, USA).

2.2. Animals

Three female horses, 10- to 15-year-old, weighting 450–500 kg, were used in the study. Animals were housed in separate indoor recovery rooms for the duration of the study and were determined to be in good health by physical examination and hematology. Except during the study, animals were fed a standard diet, alpha alpha hay in the morning (8 kg/day) (Equifioc, Molitoria Val di Serchio, Lucca, Italy) and crops in the afternoon (1.5 kg/day). They had *ad libitum* access to water. Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Pisa (authorization n° 9403 transmitted to the Italian Ministry of Health).

2.3. Experimental design

The mares received an oral single dose of tepoxalin at 10 mg/kg BW (Zubrin 200 mg tablets, Schering-Plough, Co. Summit, NJ, USA). The drug was administered in the morning, the horses were fasted for 12 h the previous night and up to 6 h after drug administration. All the animals were given the oral lyophilised formulation dissolved in 100 mL of distilled water *via* nasogastric tube. After administration, the nasogastric tube was rinsed with 1 L of distilled water to ensure complete delivery of the drug into the stomach.

Blood samples for analysis (10 mL) were collected by venipuncture from the jugular at 0 and 10 h after tepoxalin administration, and placed in collection tubes containing lithium heparin. The blood samples were centrifuged at 400 × *g* for 5 min within 30 min of collection, and the harvested plasma was stored at −20 °C and was analyzed within 14 days after collection.

2.4. Sample preparation

Detection of tepoxalin, its active acidic metabolite (RWJ-20142) and its putative metabolites in plasma were carried out by high-performance liquid chromatography, using a modification of a procedure developed by Homer et al. [8]. Briefly, 0.5 mL plasma was added in a glass test tube, followed by 1 mL 0.1 M phosphate buffer (pH 6) and 5 mL methyl-t-butyl ether. The tube was capped, vortexed for approximately 30 s, and then centrifuged to separate organic from aqueous layers. The organic layer was aspirated, transferred to another test tube, and evaporated under a N_2 stream until dry. The residue was dissolved in 200 μL mobile phase (43% 50 mM ammonium acetate buffer (CH_3COONH_4/CH_3COOH pH 5), 20% methanol, and 37% acetonitrile). Concentration standards (0, 0.1, 0.5, and 1 μg/mL) were prepared by adding appropriate amounts of tepoxalin and RWJ-20142 dissolved in methanol to plasma collected from untreated horses. Standards were subjected to the same analytical procedure as test samples. Extracted samples were poured through 0.45 μm nylon filters and 50 μL aliquots were injected into a HPLC-FL.

2.5. HPLC-FL

The high-performance liquid chromatography system consisted of a PU 980 isocratic pump attached to an AS-1550 autosampler and an 821-FP fluorimetric detector (Jasco, Essex, UK). Integration was performed using Borwin chromatographic software (version 1.21). The compounds were separated by a C_{18} column (250 mm × 4.6 mm i.d., 5 μm particle size) (Gemini, Phenomenex, Torrance, CA, USA). The flow rate of the mobile phase was 1.0 mL/min. Injection volume was 50 μL and the detector wavelengths were set at 290 nm and 440 nm as excitation and emission, respectively [11]. The chromatography was conducted at room temperature.

2.6. HPLC–MS

HPLC–MS experiments were carried out by an AB-Sciex API 4000 triple quadrupole mass spectrometer (Concord, ON, Canada), equipped with a Turbo-V electrospray (ESI) source, coupled to a Perkin Elmer 200 Series HPLC system (Waltham, MA, USA), including a binary micro pump system, a high pressure mixer, a column oven, and an auto-sampler. HPLC made use of the same column, mobile phase composition and flow rate used for HPLC-FL. Mass spectrometry was first used in full scan mode (MS), in order to investigate the presence of unknown compounds which could be involved in the metabolic pathway of tepoxalin. Afterward, tandem mass spectrometry experiments were carried out in order to elucidate the structure of those compounds. In detail, full scan acquisitions were mainly carried out according to the following parameters: positive ion mode; mass range, 250–410 Th; declustering potential (DP), 50 V; ionspray voltage (IS), 5.5 kV; curtain gas (CUR), nitrogen at 10 arbitrary units; gas source 1 (GS1), zero air at 55 arbitrary units; gas source 2 (GS2), zero air at 50 arbitrary units; source temperature (TEM), 650 °C; entrance potential (EP), 10 V. Tandem mass spectrometry experiments were carried out in both product ion scan (MS2) and precursor ion scan modes, by using 10 V as an entrance potential (EP), nitrogen as a collision (CAD) gas

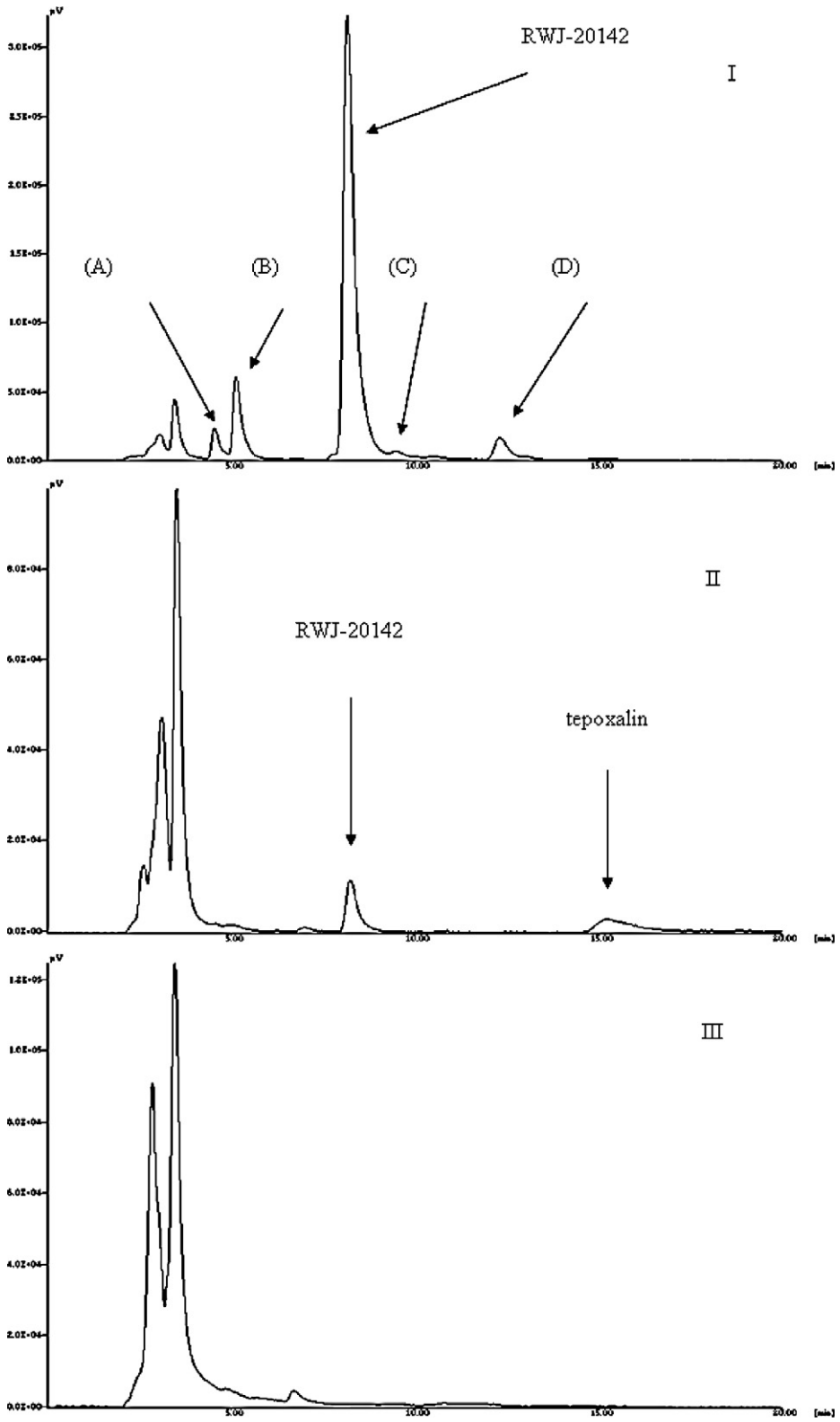

Fig. 2. (I) Chromatographic curve using plasma from the horses administered with tepoxalin (10 h); (II) chromatographic curve from fortified sample (tepoxalin, RWJ-2014, both 0.1 μg/mL) of horse plasma; (III) chromatographic curve from blank of horse plasma.

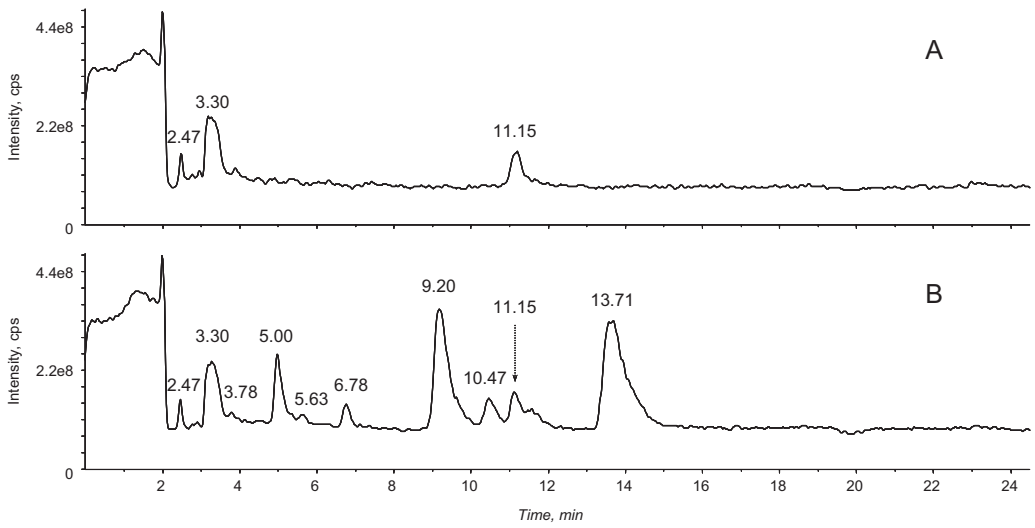

Fig. 3. Comparison between the total ion current chromatogram from horse plasma samples, as it is at 0 h (A) and 10 h after the tepoxalin administration (B).

at 5.1 mPa (operative pressure with CAD gas on), 30 eV as collision energy (CE) and keeping all the other parameters as for full scan mode acquisitions. Product ion scan mode acquisitions made use of the following precursor ions: 329, 343, 356, 357, 370, and 386 Th, while precursor ion scan mode acquisitions were performed on the ions at 325 and 339 Th.

3. Results and discussion

The HPLC method from Homer et al. [8] was modified and adapted for the present research in accordance with its intended use. In contrast to other previous HPLC studies [6,9,11], where only a single unknown peak was detected, diverse peaks were distinguished by the present method. In fact, the HPLC-FL process allowed the resolution of 4 different unknown peaks along with tepoxalin and RWJ-20142 (Fig. 2). They had a good resolution and retention times of 4.75 (A), 5.30 (B), 8.20 (RWJ-20142), 9.50 (C), 12.5 (D) and 15.20 min (tepoxalin). No peak at higher retention time was detected, even after increasing organic solvent and run time up to 1 h. Similar chromatographic profiles were obtained from three different horses.

A previous study indicated [9] that tepoxalin is totally metabolized 10 h after oral drug administration in horses (LOQ of the method 5 ng/mL). In the present study, tepoxalin was detected in negligible (trace) amounts in *in vivo* samples at 10 h (Fig. 2I). Its peak (with the highest retention time) had a squat and elongate shape. Actually, HPLC parameters were chosen in order to get an optimal resolution of the unknown peaks, even though they detrimentally affected tepoxalin peak shape that might have been overcome by increasing the amount of organic solvent in the mobile phase after

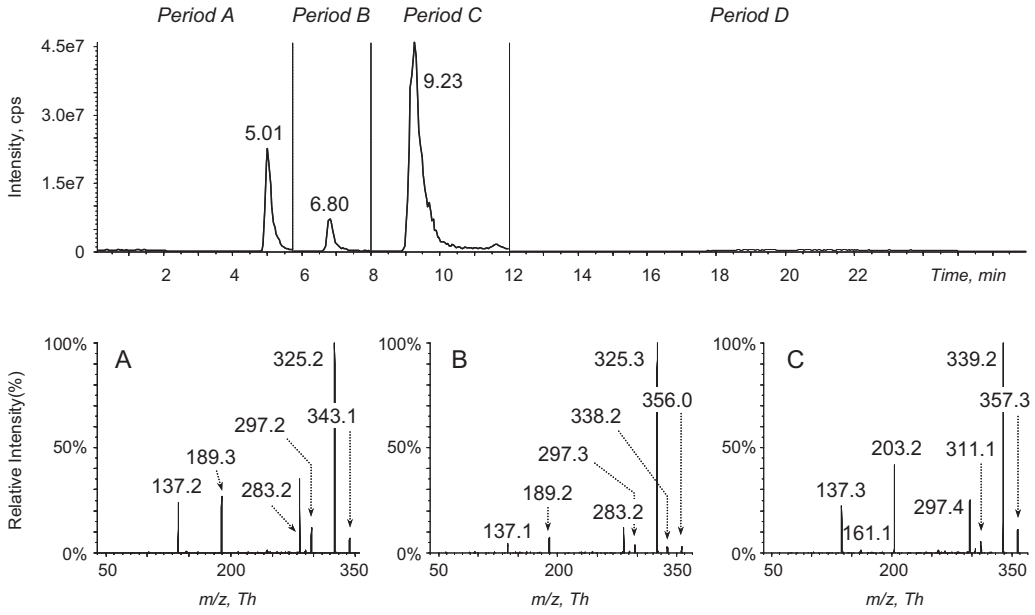

Fig. 4. Total ion current chromatogram from different MS2 experiments, performed during the same HPLC run: period A (0/5.7 min), product ion scan of *m*/*z* 343 Th; period B (5.7/8.0 min), product ion scan of *m*/*z* 356 Th; period C (8.0/12.0 min), product ion scan of *m*/*z* 357 Th; period D (12.0/27.0 min), product ion scan of *m*/*z* 386 Th. MS2 spectra (A), (B), and (C) are relative to the chromatographic peaks at 5.01 min, 6.80 min, and 9.23 min, respectively. The plasma sample used in this acquisition was withdrawn 10 h after the tepoxalin administration.

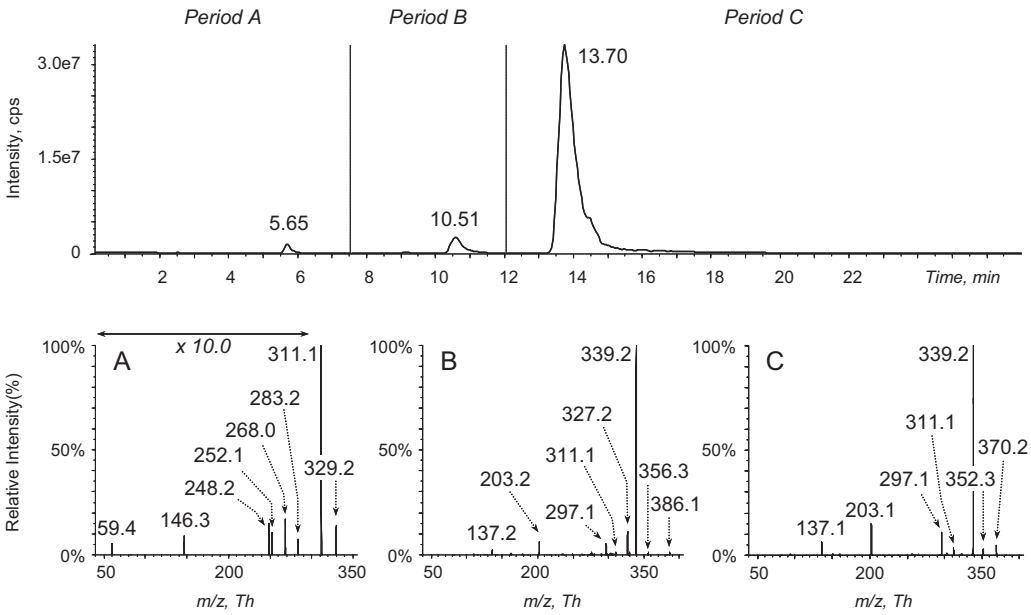

Fig. 5. Total ion current chromatogram from different MS2 experiments performed during the same run: period A (0/7.5 min), product ion scan of *m*/*z* 329 Th; period B (7.5/12.0 min), product ion scan of *m*/*z* 386 Th; period C (12/27 min), product ion scan of *m*/*z* 370 Th. MS2 spectra (A), (B), and (C) are relative to the chromatographic peaks at 5.56 min, 10.51 min, and 13.70 min, respectively. The plasma sample used in this acquisition was withdrawn 10 h after the tepoxalin administration.

the elution of the D peak. However, a simple isocratic method was preferred in order to avoid difficulties in the comparison between HPLC coupled to fluorescence and to MS, using different HPLC devices, with different specifications and dead volumes.

The samples' analysis by HPLC-MS in full scan mode, basically confirmed the general chromatographic profile shown by HPLC-FL, although different response factors and a shift in the retention times, caused by a different instrumental layout, were present (Fig. 3). That made an investigation by MS on the structures of the unknown metabolites revealed by FL possible.

A previous study [9] indicated that the concentration of metabolites (RWJ-20142 and one unknown) is highest 10 h after oral administration of the drug in horses. A comparison between plasma samples collected at 0 h and 10 h after tepoxalin administration identified in the latter sample the presence of chromatographic peaks associated to mass spectra having molecular ions at *m*/*z* 329, 343, 356, 357, 370, and 386 Th. All of these compounds contained a chlorine atom, which suggested their probable involvement in tepoxalin metabolism. Most of the MS2 spectra of these compounds showed a base peak ion at *m*/*z* 325 or 339 Th (Figs. 4 and 5); this latter ion is also the base peak in tepoxalin's MS2 spectrum (Fig. 6B). Additional experiments in precursor ion scan mode using *m*/*z* 325 or 339 Th as product ions, permitted detection of all compounds with these two ions in their MS2 spectra (Fig. 7). All these compounds had already been revealed by acquisitions in full-scan and product ion scan modes (325 ← 343 Th, 5.01 min (*II*); 325 ← 356 Th, 6.81 min (*III*); 339 ← 357 Th, 9.23 min (*IV*); 339 ← 386 Th, 10.54 min (*V*); 339 ← 370 Th, 13.70 min (*VI*)), except for one compound which had never been detected in previous experiments: 339 ← 356 Th, 11.52 min (*VII*). Interestingly, although compounds *III* and *VII* both had a $[M+H]^+$ ion at *m*/*z* 356 Th, their MS2 spectra were quite different (Fig. 8). In particular, compound *III* had *m*/*z* 325 Th as a main

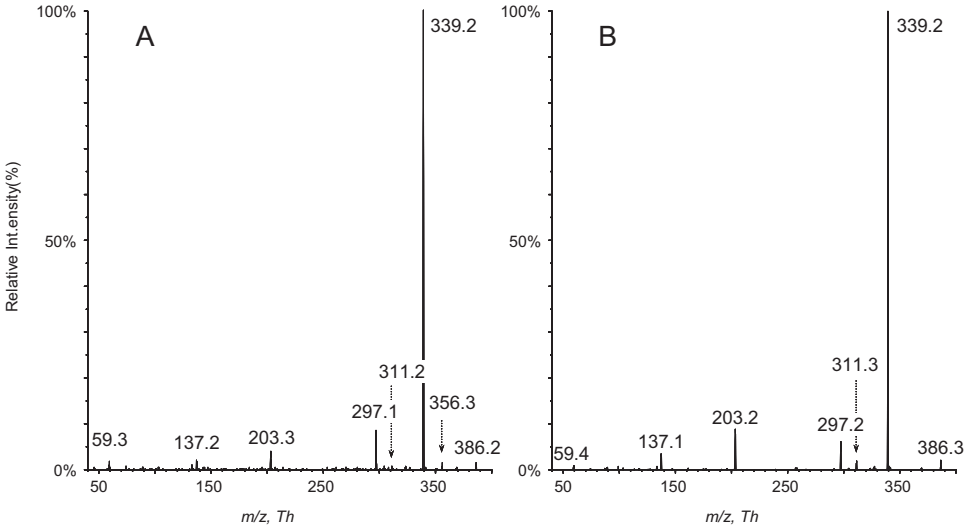

Fig. 6. MS2 spectra of compound *V* (A) and tepoxalin (B).

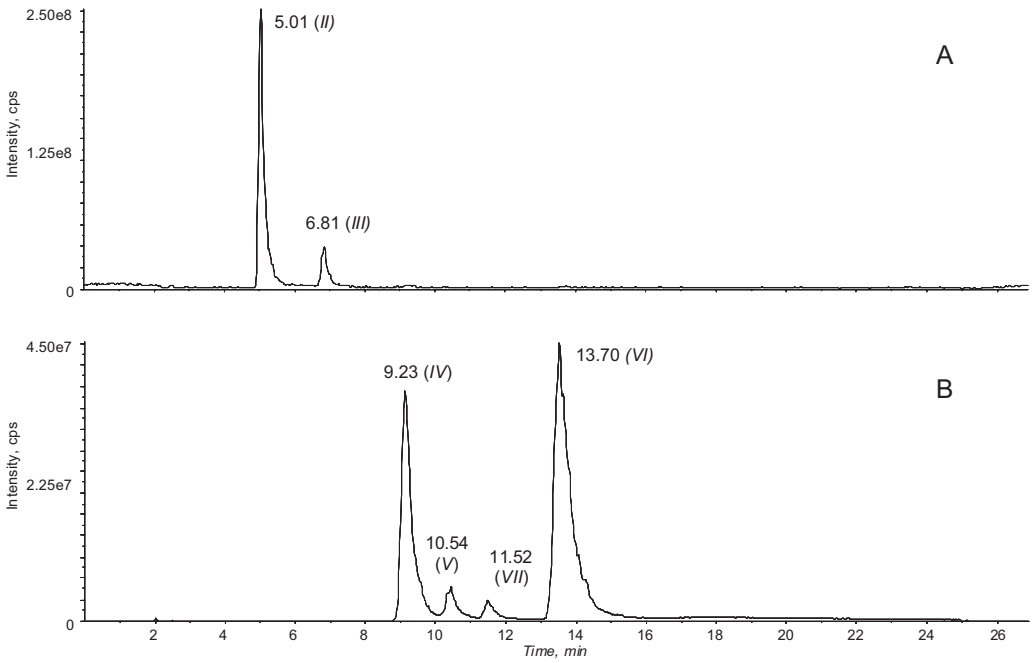

Fig. 7. Precursor ion scan chromatograms relative to the ions at *m*/*z* 325 Th (A) and at *m*/*z* 339 Th (B), from horse plasma sample collected at 10 h following tepoxalin administration.

fragment, while compound *VII* exhibited *m*/*z* 339 Th as a base peak, this is 14 mass units (CH_2) higher. This shift was confirmed by most of the other fragments (*m*/*z* 297 vs. 311, *m*/*z* 283 vs. 297, *m*/*z* 189 vs. 203 Th), suggesting that the OCH_3 group linked to one of the phenyl rings could have been replaced by an OH group. The experimental data, together with data available in the literature [12], suggested that these two ions could have the structures shown in Fig. 9A and B.

A detailed analysis of all the information collected has led to a tentative characterization of a large part of these compounds.

As an example, the complete MS2 fragmentation pattern of tepoxalin is depicted in Fig. 10. A significant contribution to the understanding of this fragmentation mechanism was provided through comparison between the product ion spectra of $[M+H]^+$ containing ^{35}Cl, *m*/*z* 386 Th, and product ion spectra of $[M+H]^+$ containing ^{37}Cl, *m*/*z* 388 Th; this revealed those fragments having a chlorine atom (Fig. 11). Actually, in the MS2 spectrum from *m*/*z* 388 Th, these fragments are located two mass units higher than the corresponding ions from *m*/*z* 386 Th (139 vs. 137 Th, 299 vs. 297 Th, 313 vs. 311 Th, and 341 vs. 339 Th).

Analogous analyses were done for compounds *II*, *III*, *IV*, *VI*, and *VIII* and the resulting structures are reported in Fig. 12, except for metabolite *V*. This was not characterized as its spectrum is very similar to that of tepoxalin (Fig. 6). This similarity suggests that such a compound could be an isomer of tepoxalin (*I*) [12], with structural differences accounting for the difference in the retention time with respect to the latter (10.51 vs. 17.72 min).

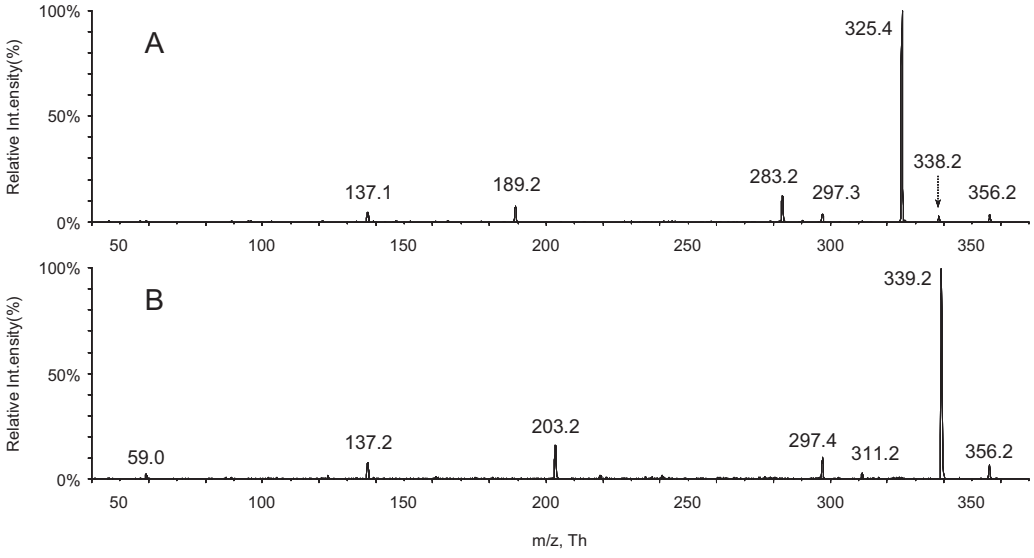

Fig. 8. Comparison between the MS2 spectra of two different compounds both having 355 as a molecular weight: compound *III* (A) and compound *VII* (B).

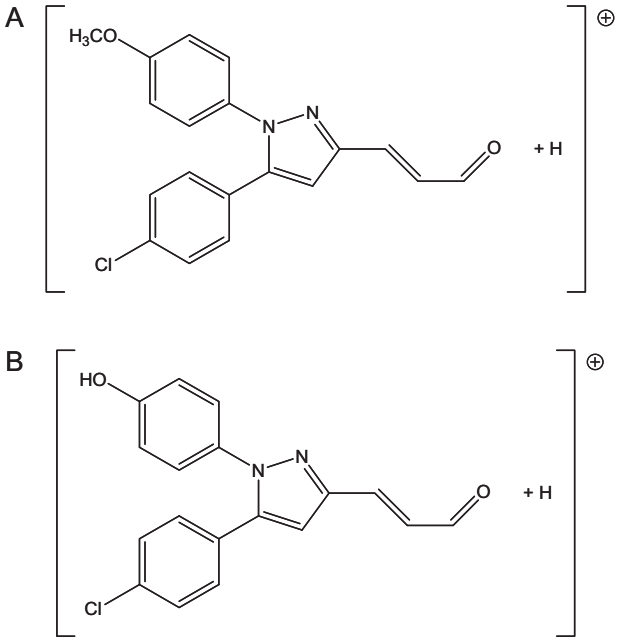

Fig. 9. Tentative structures of the fragment ions at *m*/*z* 339 Th (A) and 225 Th (B), which are the base peak in the MS2 spectra of several tepoxalin metabolites.

Total ion current chromatogram in Fig. 3 also exhibits a small peak at 5.63 min, associated with a spectrum containing the $[M+H]^+$ ion at *m*/*z* 329 Th, with an isotopic cluster informing about the presence of a chlorine atom. The relative MS2 spectrum (Fig. 5A) had a base peak at *m*/*z* 311 Th, which is consistent with the loss of a water molecule. Unfortunately, except for the ions at *m*/*z* 311 Th, the other fragments (expanded 10 folds in Fig. 5A) exhibited an intensity that was always lower than 2%. Moreover, the low concentration of this compound in horse plasma could result in an inability to detect some important fragments useful for full determination of the structure. In any case, based on extrapolation from information on previously described compounds, *VIII* in Fig. 12 is proposed as a tentative structure.

It is conceivable that some of these molecules could be the products of impurities in Zubrin. However, Zubrin is a commercial drug and as such, the manufacturers are obliged to comply with regulations and guarantee their drug is of a high purity. Additionally, according to a former manuscript [12], tepoxalin impurities only result if the active ingredient is exposed to particular extremes in temperature, light or environmental conditions. Hence, the possibility of degradation products influencing these results is considered highly unlikely.

As far as structure *V* (Fig. 12) is concerned, this is the first time that an *in vivo* isomeric metabolite is reported for tepoxalin; this could play an important role in the pharmacokinetics of this drug. It is known that isomers can play an important role in the pharmacology of several drugs, in many instances, isomers have been shown to drastically influence the drug's pharmacokinetic/dynamic features (i.e. [13]). An understanding of their influence is therefore quite important for the evaluation of the correct dose to dispense.

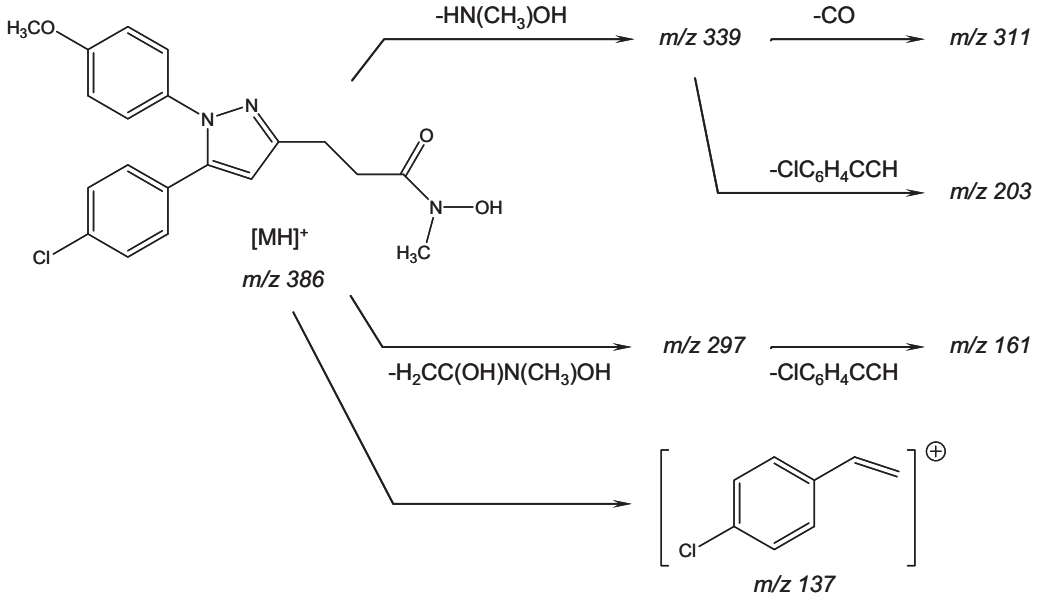

Fig. 10. Fragmentation pattern of tepoxalin.

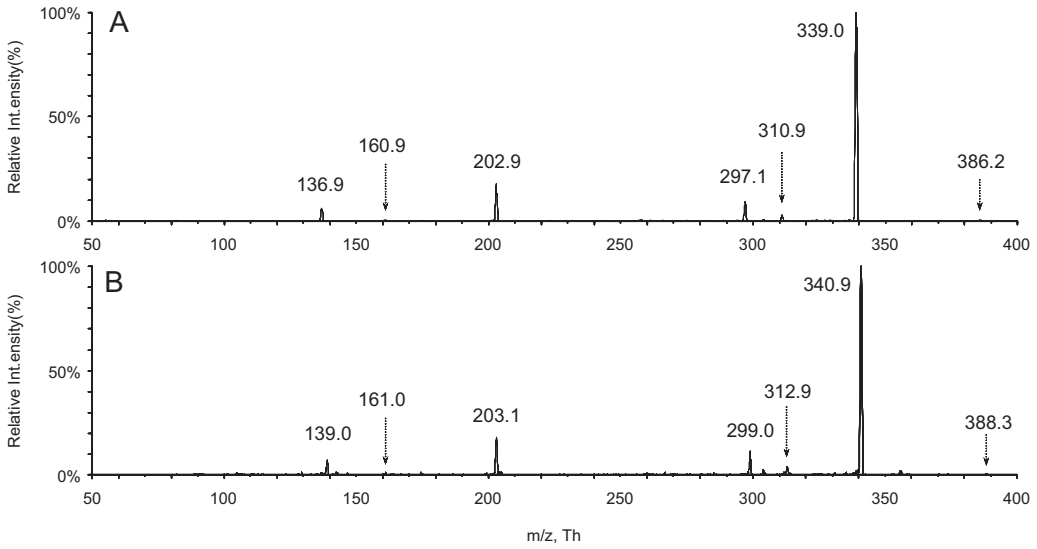

Fig. 11. The comparison between product ion spectra of $[M+H]^+$ containing ^{35}Cl, *m*/*z* 386 Th (A), and containing ^{37}Cl, *m*/*z* 388 Th (B), pointing out fragments including a chlorine atom.

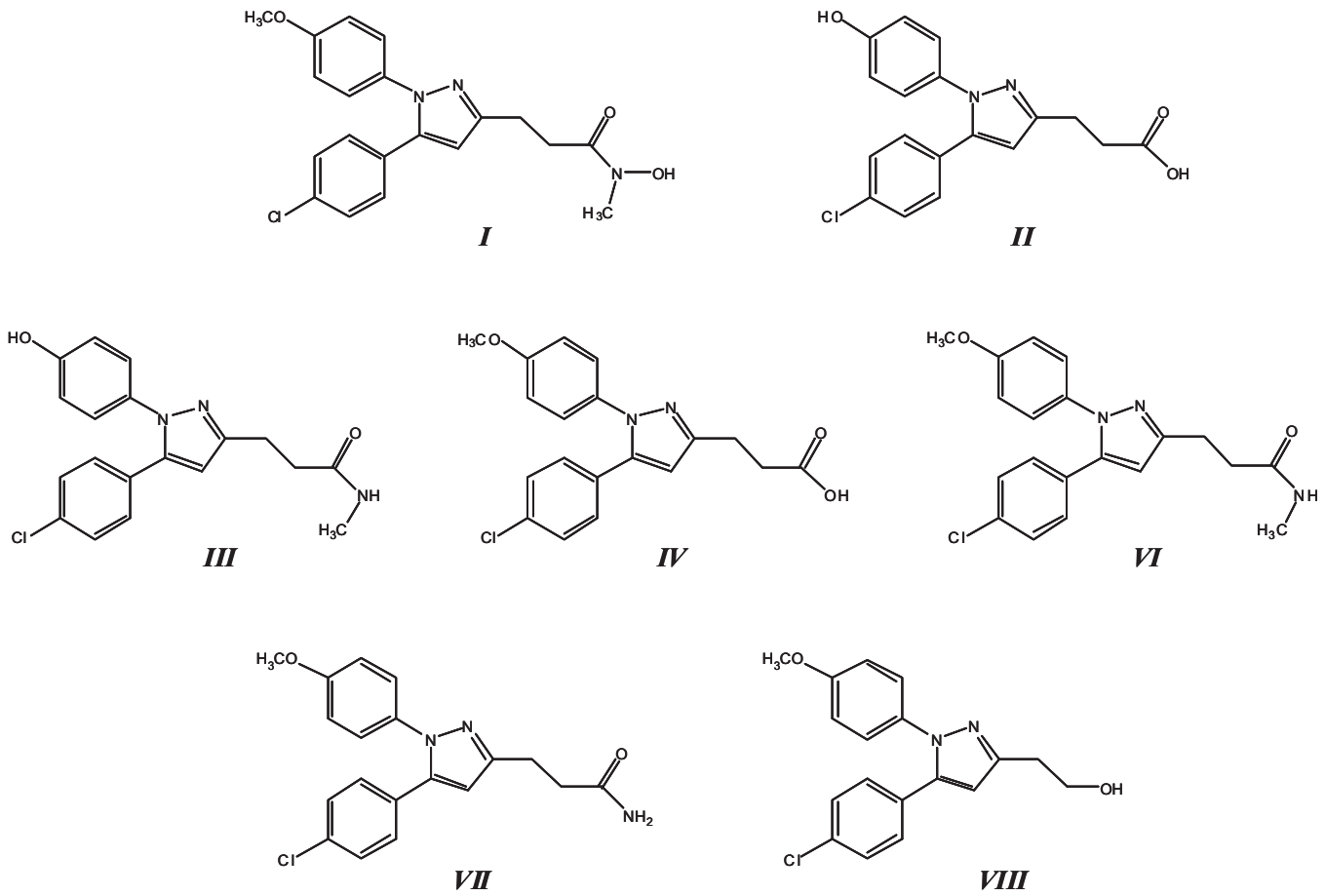

Fig. 12. Structures for tepoxalin and some of its metabolites: *I*, tepoxalin, MW 385; *II*, MW 342; *III*, MW 355; *IV*, RWJ20141, MW 356; *VI*, MW 369; *VII*, MW 355; *VIII*, MW 328.

Hence it would be interesting to evaluate the isomers' effectiveness in terms of COX and 5-LO inhibitor activities.

4. Conclusions

This study describes for the first time the main plasma metabolic pattern of tepoxalin 10 h following its oral administration in horses. Previous studies were focused on the main metabolite (RWJ-20142), without considering the others. In the present study only one extraction method was used; because of this, some metabolites possessing different proteolytic properties to the parental drug, may not have been detected. Hence, further investigations are still necessary to elucidate the full metabolic pattern of this drug.

Mass spectrometry allowed the tentative characterization of a several tepoxalin metabolites, some of them well known and already described in the literature as pharmaceutical impurities, while some others, to the best of our knowledge, have never been described. Unfortunately, no data on their pharmacological activities can be offered, but these findings pave the way for further research on tepoxalin. Extrapolation of metabolism data from horses and other animal species could be an important tool to correlate *in* vivo pharmacokinetic and metabolic data with the effectiveness/toxicity of the drug in different animal species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.03.028.

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