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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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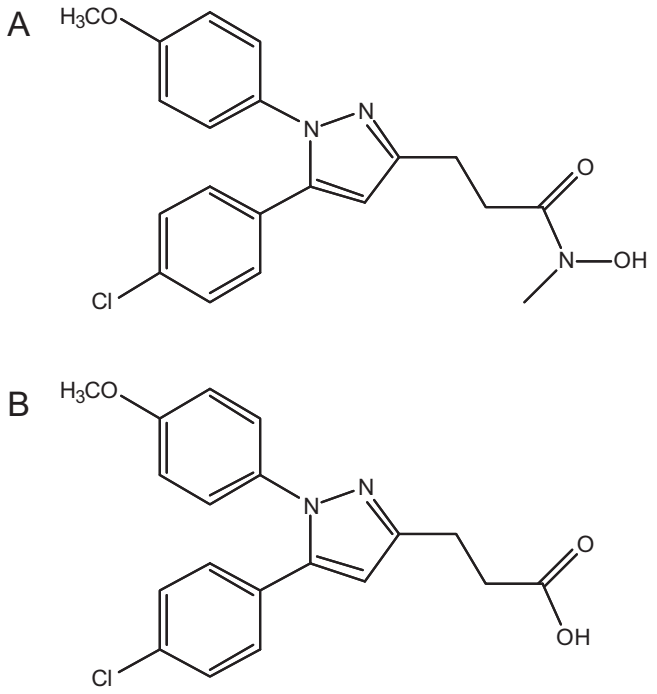

Fig. 1. Molecular structure of (A) tepoxalin, (B) its active metabolite (RWJ-20142).

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