Comparative Biochemistry and Physiology, Part C 161 (2014) 7–14

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

In vitro hepatic microsomal metabolism of meloxicam in koalas (*Phascolarctos cinereus*), brushtail possums (*Trichosurus vulpecula*), ringtail possums (*Pseudocheirus peregrinus*), rats (*Rattus norvegicus*) and dogs (*Canis lupus familiaris*)

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ARTICLE INFO

Article history:
Received 12 October 2013
Received in revised form 9 December 2013
Accepted 9 December 2013
Available online 15 December 2013

Keywords:
Marsupials
Rat
Dog
Meloxicam
Microsome
Clearance
Metabolism

ABSTRACT

Quantitative and qualitative aspects of *in vitro* metabolism of the non-steroidal anti-inflammatory drug meloxicam, mediated *via* hepatic microsomes of specialized foliage (*Eucalyptus*) eating marsupials (koalas and ringtail possums), a generalized foliage eating marsupial (brushtail possum), rats, and dogs, are described. Using a substrate depletion method, intrinsic hepatic clearance (*in vitro* Cl_{int}) was determined. Significantly, rates of oxidative transformation of meloxicam, likely mediated *via* cytochromes P450 (CYP), were higher in marsupials compared to rats or dogs. The rank order of apparent *in vitro* Cl_{int} was brushtail possums ($n = 3$) (mean: 394 μL/min/mg protein), >koalas ($n = 6$) (50), >ringtail possums ($n = 2$) (36) (with no significant difference between koalas and ringtail possums), >pooled rats (3.2) > pooled dogs (in which the rate of depletion, as calculated by the ratio of the substrate remaining was <20% and too slow to determine). During the depletion of meloxicam, at a first-order rate constant, 5-hydroxymethyl metabolite (M1) was identified in the brushtail possums and the rat as the major metabolite. However, multiple hydroxyl metabolites were observed in the koala (M1, M2, and M3) and the ringtail possum (M1 and M3) indicating that these specialized foliage-eating marsupials have diverse oxidation capacity to metabolize meloxicam. Using a well-stirred model, the apparent *in vitro* Cl_{int} of meloxicam for koalas and the rat was further scaled to compare with published *in vivo Cl*. The closest *in vivo Cl* prediction from *in vitro* data of koalas was demonstrated with scaled hepatic $Cl_{(total)}$ (average fold error = 1.9) excluding unbound fractions in the blood and microsome values; whereas for rats, the *in-vitro* scaled hepatic $Cl\, f_{u(blood,\ mic)}$, corrected with unbound fractions in the blood and microsome values, provided the best prediction (fold error = 1.86). This study indicates that eutherians such as rats or dogs serve as inadequate models for dosage extrapolation of this drug to marsupials due to differences in hepatic turnover rate. Furthermore, as *in vivo Cl* is one of the pharmacokinetic indexes for determining therapeutic drug dosages, this study demonstrates the utility of *in vitro* to *in vivo* scaling as an alternative prediction method of drug *Cl* in koalas.

1. Introduction

Meloxicam, a non-steroidal anti-inflammatory drug (NSAID), is a preferential inhibitor of inducible cyclooxygenase-2 (COX-2) and has anti-inflammatory, analgesic, and antipyretic properties with reportedly lower ulcerogenic potency than non-specific COX inhibitors (Engelhardt et al., 1995; Engelhardt, 1996). Meloxicam is used for the treatment of osteo- and rheumatoid arthritis in humans (Gates et al., 2005), and as an anti-inflammatory and analgesic for domestic species (such as dogs and horses). The dosage used for humans and for some domestic species has been determined *via* species-specific pharmacokinetic studies (Lees et al., 1991; Schmid et al., 1995a; Busch et al., 1998). Accordingly, meloxicam is generally reported to have good oral absorption, high plasma protein binding resulting in a narrow volume of distribution and limited renal excretion of the parent drug and slow clearance (*Cl*); although *Cl* demonstrates some variability among species (Lees et al., 1991; Schmid et al., 1995a; Busch et al., 1998). Meloxicam is reported to undergo extensive hepatic metabolism, primarily *via* oxidation, which is mediated *via* cytochromes P450 (CYP) enzymes, and this pathway appears consistent between species (Busch et al., 1998). Knowledge of pharmacokinetics, particularly *Cl*, is essential to estimate the dosage to sustain desired plasma concentration of the drug; however it is lacking for many wild and exotic species for which the dosage is usually extrapolated from that used for rats, dogs or humans, or based on anecdotal observations. Meloxicam is a popular analgesic

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http://dx.doi.org/10.1016/j.cbpc.2013.12.002

administered to marsupials in Australia, such as koalas, with the recommended dosage similar to that of dogs or humans (Blanshard and Bodley, 2008). In contrast to the pharmacokinetic profile in eutherian species, it was recently demonstrated that meloxicam administration in koalas had low oral and subcutaneous plasma concentrations due to an extremely rapid plasma *Cl* (0.44 L/h/kg) (Kimble et al., 2013a) relative to rats (0.015 L/h/kg) (Busch et al., 1998), dogs and humans (both approximately 0.01 L/h/kg) (Busch et al., 1998). The rapid *Cl* in koalas was presumed to result from a superior intrinsic hepatic clearance rate (Cl_{int}), especially *via* oxidative metabolism (Kimble et al., 2013a). The aim of the present study was to investigate *in vitro* Cl_{int} of meloxicam in koalas, utilizing hepatic microsomes, in order to confirm the *in vivo Cl* (compared with *in vitro* to *in vivo* scaling value) and to compare koalas' Cl_{int} with that of other marsupials, such as brushtail possums and ringtail possums. As rats and dogs are conventional models for *in vitro* and *in vivo* drug metabolism studies for human pre-clinical studies (Zuber et al., 2002), meloxicam *in vitro* Cl_{int} for these species was also investigated.

2. Materials and methods

2.1. Chemicals

Meloxicam and piroxicam (the latter used as the internal standard, [IS] for liquid chromatography), NADP, glucose 6-phosphate dehydrogenase and glucose 6-phosphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Liquid chromatography (LC) grade solvents were obtained from Analytical Science (Sydney, NSW, Australia).

2.2. Preparation of liver microsomes

Hepatic microsomes from pooled Sprague Dawley male rats and from pooled male beagle dogs were purchased from Sigma-Aldrich (product number M 9066) and BD Biosciences (Woburn, MA, USA; lot number 00269), respectively, and stored at −80 °C prior to use. Recently deceased (<24 h) koalas ($n = 6$) were transported chilled to our institution where livers were quickly removed and microsomes extracted. Liver harvesting for brushtail possums ($n = 3$) and ringtail possums ($n = 2$) occurred immediately after death and was transported to our institution in ice within 2 h, where microsome harvesting occurred and stored at −80 °C. All livers were collected opportunistically from animals euthanized with pentobarbital by veterinarians for reasons independent of this study (such as trauma from vehicle strikes or feral animal attacks) and with no clinical signs, or gross organ appearance, of hepatic disease. Microsomes from livers were extracted and prepared according to a method described previously (Hill, 2003), with some modifications. Briefly, hepatic tissues were homogenized at 4 °C in 3 volume of a buffer containing 0.1 M Tris-Cl of pH 7.4 with, 10 mM EDTA and 150 mM KCl. The homogenate was then separated by differential centrifugation (12,500 *g* for 15 min; 19,000 *g* for 20 min; 105,000 *g* for 70 min) at 4 °C. The final pellet, the microsomal fraction, was subsequently washed and resuspended in a buffer of 50 mM Tris-Cl of pH 7.4 with 10 mM EDTA and 20% glycerol. Aliquots (~250 μL) of microsomal fractions were stored at −80 °C. Protein concentrations of the microsomal fractions were determined using the Bradford assay kit (Bio-Rad, Hercules, CA, USA) and standardized with bovine serum albumin.

2.3. Microsomal experimentation

Meloxicam (1.25 μM) was preincubated in 2 mL of 0.1 M phosphate buffer (pH 7.4) containing a NADPH regenerating system (1 mM NADP, 0.8 U glucose 6 phosphate dehydrogenase and 3 mM glucose 6 phosphate) and 3 mM $MgCl_2$, in an open air shaking water bath at 37 °C for 5 min. The enzymatic reaction was then initiated by adding a predetermined concentration of microsomal protein (0.5 mg/mL for koalas and both species of possums; 1 mg/mL for rats and dogs). During the incubation, 200 μL aliquots were removed at time (t) = 0, 2.5, 5, 7.5, and 10 min for brushtail possums ($n = 3$); 0, 5, 10, 15, 20 (koalas only), and 30 min for koalas ($n = 6$) and ringtail possums (n = 2); 0, 5, 15, 30, 45, and 60 min for the rat and dog. Each extracted aliquot was mixed with 125 μL of ice-cold methanol (which also contained 5 μM of IS) to deactivate the reaction. The resultant mixture was vortexed and centrifuged at 14,000 *g* for 10 min, and the supernatant was either stored at −80 °C or directly injected to the HPLC system for analysis. In addition, to determine stability of meloxicam, incubation of the drug without NADPH, in which the corresponding volume was substituted by buffer, was undertaken for time points up to and including 60 min for each species. All samples were prepared and analyzed in duplicate. To identify the structure of any metabolites, meloxicam concentrations of 1.25 μM underwent additional incubation containing a NADPH regenerating system with pooled hepatic microsomes of 1 mg/mL of each species for 0, 30, and 60 min, and were analyzed by liquid chromatography–mass spectrometry (LC–MS).

2.4. Microsomal binding and blood–plasma (B/P) ratio in the koala and rat

Meloxicam (1.25 μM) was incubated with 0.5 mg/mL of pooled koala microsomes ($n = 3$) or 1 mg/mL pooled rat microsomes in 1 mL of 0.1 M phosphate buffer (pH 7.4) containing 3 mM $MgCl_2$, in an open air shaking water bath at 37 °C for 30 min. The same mixtures without microsomal protein served as controls. After incubation, both samples and controls were transferred to the reservoir of the ultrafiltrate device (10 kDa) (Millipore, Billerica, MA, USA) and centrifuged (1500 *g*) for 15 min at 37 °C. Upon completion, the filtrate portion was analyzed *via* HPLC. The filtrate portion of the control was used for determination of recovery of meloxicam which was ~65%. All experimentation was in triplicate. The unbound fraction of microsomes, $f_{u(mic)}$, was expressed as concentration ratio between samples *vs.* control ($C_{sample}/C_{control}$).

For koalas, the meloxicam B/P ratio was determined according to method previously described (Yu et al., 2005). Briefly, final concentrations of 0.1 and 0.2 μg/mL of meloxicam (comparable to the C_{max} obtained previously by Kimble et al., 2013a) were added to 1 mL of fresh pooled whole blood of koalas ($n = 2$) and incubated at 37 °C for 0, 10, 30, and 60 min. After incubation, the plasma was separated from whole blood and concentrations of meloxicam in the separated plasma were measured (Cp). Prior to the assay, the hematocrit (Hct) value of the koala blood was determined with an automated hematology analyzer, Sysmex XT-2000i (Kobe, Japan) by an accredited veterinary pathology lab. For a control that represented the whole blood concentration (ref Cp) the same concentration of meloxicam (0.1 and 0.2 μg/mL) was added to 1 mL of blank koala plasma and incubated at 37 °C for 0, 10, 30, and 60 min. The B/P ratio was determined by ref Cp/Cp. In addition, the theoretical B/P ratio was calculated in koalas using the following equation:

$$B/P = 1 + Hct \times (f_{u(plasma)} - 1)$$

where $f_{u(plasma)}$ is the unbound fraction in the plasma which is 0.0183 in koalas (Kimble et al., 2013a). As <10% of meloxicam is recognized to penetrate rat red blood cells (Busch et al., 1998), the B/P ratio was calculated from the above equation. Hct value and $f_{u(plasma)}$ used for rats were 0.46 L/L (Zou et al., 2012) and ~0.004 (Busch et al., 1998), respectively.

2.5. Calculations

The Cl_{int} was estimated by the substrate depletion method using *in vitro* $t_{1/2}$ approach (Obach et al., 1997; Obach, 1999). Briefly, using the peak ratio of meloxicam/IS at $t = 0$ as 100% of substrate, the peak ratio of the other time points was converted to a percentage of the