HPLC Method with Solid-Phase Extraction for Determination of (R)- and (S)-Ketoprofen in Plasma without Caffeine Interference: Application to Pharmacokinetic Studies in Rats

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Received 26 July 2013; revised 25 October 2013

A fast and reproducible high-performance liquid chromatography method has been developed for the determination of (R)- and (S)-ketoprofen. Ketoprofen enantiomers were determined in plasma samples (50 µL), after solid-phase extraction, using diclofenac as internal standard. Analyses were performed on a (S, S)-Whelk-O 1 stainless steel column (5 µm, 250 x 4.6 mm) using hexane–ethanol–acetic acid (93:7:0.5, v/v/v) as the mobile phase and detection at 254 nm. The method was selective for ketoprofen enantiomers in the presence of caffeine and endogenous plasma compounds. Standard curves were linear (R² > 0.999) over the concentration range of 0.25–12.50 and 0.25 µg/mL was taken as the limit of quantification. The intra- and interday precision (relative standard deviation) values were <15.0% and the accuracy (relative error) was within ±12.0% at 1.0, 5.0 and 10.0 µg/mL. Enantiomer recoveries yielded 100.0 ± 15%. No significant differences were determined in plasma samples stored at room temperature for 24.0 h, after two freeze–thaw cycles, and between 0 and 4 weeks at −20°C (P > 0.05). The validated method was successfully applied in determination of (S)-ketoprofen in Wistar rats after oral administration of 3.2 mg/kg of (S)-ketoprofen alone or 3.2 mg/kg of (S)-ketoprofen + 17.8 mg/kg of caffeine.

Introduction

(R, S)-Ketoprofen is a clinically important non-steroidal anti-inflammatory drug (NSAID) widely used in veterinary and human medicine to treat arthritis-related inflammatory pain. The drug has a chiral center at the α-carbon of the carboxyl group. Therefore, it exists as the (R)- and (S)-enantiomeric forms (Figure 1). Only the (S)-enantiomer selectively inhibits the cyclooxygenase (COX-2), an enzyme involved in the arachidonic cascade that generates inflammatory mediators of the prostaglandin group, while the (R)-enantiomer has no pharmacological activity related to COX-2 inhibition (1).

The pharmacokinetics of ketoprofen enantiomers has been reported for several animal species: calves (2), camels (3), cats (4), elephants (5), goats (6), growing pigs (7), horses (8), mice (9), piglets (10), rats (11–13) and sheep (14). Unrelated to dose, there is evidence that (R, S)-ketoprofen undergoes chiral inversion from (R)- to (S)-enantiomer in rats (12, 13), while the drug can show bidirectional inversion with a lesser extent from (S)- to (R)-enantiomer in mice (9). On the other hand, most analytical methods for the determination of (R)- and (S)-ketoprofen in biological fluids include the use of high-performance liquid chromatography (HPLC) (15), with liquid–liquid extraction procedures, while just few enantioselective methods including solid-phase extraction have been reported. Aboul-Enein et al. (16) published a procedure involving solid-phase and liquid–liquid extractions, as well as a derivatization step, using 1 mL of horse plasma sample together with NMR/MS analysis for the identification of 9-aminophenanthrene derivatives with run time 20 min. Ameyibor and Stewart (17) and Boisvert et al. (18) reported a simple relative analysis using 1 mL of human serum and plasma, respectively, which is still a disadvantage for the determination of (R)- and (S)-ketoprofen in small animal species. Mustonen et al. (7) and Eichhold et al. (19) described a sensitive method for the determination of ketoprofen enantiomers with liquid chromatography-tandem mass spectrometry but the disadvantage of the expensive investment in equipment. None of these methods involved the determination of ketoprofen enantiomers in the presence of caffeine.

It is well known that caffeine is able to potentiate the analgesic activity of other drugs in humans (20) and the antinociceptive effect in animals (21–23). Caffeine by itself does not produce any antinociceptive effect at lower doses than 50 mg/kg in rats and mice (21); however, when it is administered with some NSAIDs, it modifies their antinociceptive effect. Medina et al. (25) demonstrated that when six ibuprofen doses (5.6–100.0 mg/kg) were concomitantly administered with three caffeine doses (10.0, 17.8 or 31.6 mg/kg), the antinociceptive efficacy was increased in all combinations. The antinociceptive efficacy, showed by the combination of ibuprofen + 17.8 mg/kg of caffeine, was greater than the antinociceptive efficacy of morphine, ibuprofen or acetysalicylic acid alone. Then again, Díaz-Reval et al. (22) evaluated five ketoprofen doses (1.0–100.0 mg/kg) alone and combined with two caffeine doses (10.0 or 17.8 mg/kg) thus found that the best combination was 3.2 mg/kg of ketoprofen + 17.8 mg/kg of caffeine with an 18-fold potency increase, as compared with the same dose of ketoprofen alone. It has been suggested that this potentiation could be due to an increase on drug bioavailability by enhancing its absorption or impairing its elimination (24). Also, it has been reported that caffeine can modify gastric acidity as well as gastric and hepatic blood flows (25, 26). If caffeine decreases the liver blood flow, it may reduce hepatic clearance (27). These mechanisms could help to explain the potentiation obtained with the combination of NSAIDs + caffeine; however, no information about a possible
pharmacokinetic interaction between (S)-ketoprofen and caffeine has been published.

Since small laboratory animals as rats and mice are easily taken as animal models to evaluate nociception or pain, and as plasma concentration–time profiles help to interpret the pharmacological respond in function of some ketoprofen pharmacokinetic mechanism, a selective and reliable chromatographic method is necessary to determine (S)-ketoprofen concentrations after its oral administration to rats, alone or in combination with caffeine. The purpose of the present study was to develop and validate an HPLC method for plasma determination of ketoprofen enantiomers, without caffeine interference, using a single solid-phase extraction step and a microvolume of plasma sample (50 µL). The method was applied for the quantification of (S)-ketoprofen in samples arising from two pharmacokinetic studies in rats after oral administration of 3.2 mg/kg of (S)-ketoprofen alone or 3.2 mg/kg of (S)-ketoprofen + 17.8 mg/kg of caffeine.

Experimental
Chemicals, reagents and instrument
Analytical standards of (R, S)-ketoprofen, (S)-ketoprofen, diclofenac and caffeine from Sigma Chem Co. (St. Louis, MO, USA) were used. Acetonitrile, ethanol, hexane, methanol and phosphoric acid HPLC-grade were acquired from J.T. Baker Mexico and acetic acid analytical grade from E. Merck Mexico. Water HPLC-grade (18 Ω) was obtained by purifying distilled water in a Milli-Q filtration system (Millipore, Bedford, MA, USA). The mobile phase was filtered through a 0.45-µm PTFE membrane (Sun Sri, Rockwood, TN, USA) and degassed in an ultrasonic bath (Branson Ultrasonic Corp., Eagle Road, Danbury, CT, USA) during 15 min prior to use.

The chromatographic system consists of a Perkin Elmer Series 200 HPLC (Norwalk, CT, USA) equipped with a binary pump, a manual injector with a 20-µL loop, and a variable-wavelength UV 785 Model Detector (Applied Biosystems). PE Nelson-Turbochrom software was used for acquisition and data processing.

Calibration standards and quality control samples
Stock solutions of (R, S)-ketoprofen (0.5 mg/mL) and the internal standard, diclofenac (1.0 mg/mL) were prepared in ethanol and stored at 4°C. Rat plasma calibration standards of (R)- and (S)-ketoprofen were prepared by spiking appropriate aliquots of the stock solution of (R, S)-ketoprofen (50:50 racemic mixture) to drug-free rat plasma to give final concentrations ranging from 0.25 to 12.50 µg/mL for each enantiomer. Quality control (QC) samples at concentrations of 1.0, 5.0 and 10.0 µg/mL were prepared by adding the appropriate stock solutions to drug-free rat plasma. Aliquots of QC samples (50 µL) were placed into polypropylene tubes and used freshly or stored at −20°C until used for analysis.

Sample preparation
Samples were extracted by passing them through a preconditioned Strata-X 33 µm polymeric reversed phase cartridge (Phenomenex, Torrance, CA, USA) with the aid of a vacuum device (Vac-Elute, Speed Mate 10, Applied Separations). The cartridges were preconditioned by flushing them with 2 mL of methanol and 1 mL of water. To 50 µL of either blank plasma or calibration standards or QC samples, 100 µL of a 85% phosphoric acid/water (1:10) mixture and 10 µL of internal standard solution (diclofenac, 100 µg/mL) were added. After vortex mixing, the samples were passed through the cartridges and then washed with 600 µL of a water:methanol (95:5, v/v) mixture. The enantiomers and the internal standard were eluted with 1 mL of an acetonitrile:methanol (50:50, v/v) mixture. The eluate was evaporated to dryness on a water bath at 37°C under a gentle stream of nitrogen. The residue was reconstituted in 50 µL of mobile phase and 30 µL of it were injected into the HPLC system for analysis.

HPLC conditions
The separation was performed on a (S, S)-Whelk-0 1 stainless steel column, 5 µm particle size, 250 × 4.6 mm (Regis Technologies Inc., Morton Grove, IL, USA) protected by a Security Guard column packed with CN (cyanopropyl silica) material, 4.0 × 3.0 mm (Phenomenex®, Torrance, CA, USA). The mobile phase consisted of a mixture of hexane–ethanol–acetic acid (93:7:0.5, v/v/v) and the flow rate was 1.5 mL/min. Detection wavelength was set at 254 nm using an attenuation of 0.001 a.u.f.s. All analyses were performed at room temperature (25°C).

Validation procedures
The method was validated according to the FDA guidelines for validation of bioanalytical methods (28).

Selectivity
To determine the selectivity of the method, rat blank plasma, alone and spiked with known amounts of (R, S)-ketoprofen,
after two freeze–thaw cycles. A third set was stored at room temperature for 24.0 h. Another set was analyzed with 1.0 and 10.0 mg/L of each enantiomer versus (R)- or (S)-ketoprofen/diclofenac.

Three plasma calibration curves in a concentration range of 0.25–12.50 μg/mL of each enantiomer were determined. Standard calibration curves were generated for each enantiomer by plotting peak-area ratio of (R)- or (S)-ketoprofen/diclofenac against concentration levels along with a standard curve, on three different days. The relative standard deviation (RSD), i.e., coefficient of variation (CV), served as a precision measure. The CV should be <15%, except at limit of quantification (LOQ) where it should not exceed 20%.

The accuracy of the method was determined, on the above samples, by comparing the means of the found (R)- or (S)-ketoprofen concentrations with the nominal concentrations. QC samples of each enantiomer were analyzed on the same day, in triplicate, 95% confidence interval (CI95%) for the intercept and the determination coefficient ($R^2$).

**Calibration curves and linearity**

The relative errors (RE) should be within 20%, except at LOQ. The absolute recovery (AR) of (R)- and (S)-ketoprofen was determined by extracting QC samples of the drug by the proposed method (n = 6). The peak-areas obtained were compared with data obtained after direct injection of non-extracted (R)- and (S)-ketoprofen standard solutions in mobile phase, at the same concentration levels.

**Precision, accuracy, limit of quantification and limit of detection**

The intraday precision of the assay was determined by analyzing, on the same day, a set of QC samples of (R)- and (S)-ketoprofen (n = 6) along with a standard calibration curve. The interday precision was assessed by performing the analysis of six replicates QC samples of (R- and (S)-ketoprofen at the same concentration levels along with a standard curve, on three different days. The relative standard deviation (RSD), i.e., coefficient of variation (CV), served as a precision measure. The CV should be <15%, except at limit of quantification (LOQ) where it should not exceed 20%.

The accuracy of the method was determined, on the above samples, by comparing the means of the found (R)- or (S)-ketoprofen concentrations with the nominal concentrations. QC samples of each enantiomer were analyzed on the same day (intraday accuracy) or in three different days (interday accuracy). The mean value of RE should be within ±15% of the nominal concentration, except for the LOQ where it should not exceed 20%.

**Pharmacokinetic study**

The validated method was applied for the determination of (S)-ketoprofen in plasma samples obtained in two pharmacokinetic studies in rats after the administration of (S)-ketoprofen alone and after the concomitant administration of (S)-ketoprofen + caffeine. Male Wistar rats (Crl(W)BR) weight 180–200 g from our breeding (Cinvestav–Sede, Mexico) were used in this study. Rats were maintained under controlled environmental conditions at 22°C, under a 12-h light/dark cycle, and provided with standard chow (Purina Laboratory Rodent Diet 5001) as well as water ad libitum. Twelve hours before the experiment, food was withheld; however, rats had free access to water. The experiment was performed during the light phase, and animals were used only once. All experimental procedures and protocols were approved by the local Institutional Animal Care and Use Committee (SIACUAL Cinvestav–Sede Zacatecas, Av. Instituto Politecnico Nacional 2508 Col. San Pedro Zacatecas, Mexico DF, CP 07360; Approval No.: 444-08; valid until 3 November 2013) in accordance with the Mexican Federal Regulations for the care and use of laboratory animals, Mexican Ministry of Health (NOM-062-ZOO-1999) and adhere to the Guide for Care and Use of Laboratory Animals, Washington, DC (30).

The number of experimental animals was kept to a minimum. Two groups of six rats were used in the pharmacokinetic studies to analyze a possible pharmacokinetic interaction. The day of the study, rats were slightly anesthetized with isoflurane, and the caudal artery was cannulated with a PE-10 cannula, i.d. 0.28 mm, o.d. 0.61 mm (Clay Adams, Parsippany, NJ, USA) connected to a PE-50 cannula, i.d. 0.58 mm, o.d. 0.96 mm. The cannula was kept patent with heparin saline solution and stoppered with a needle. Rats were allowed to recover from anesthesia and a 0.2 mL of blood sample was drawn from each animal before the drug(s) administration, to serve as a control. At zero time, rats were treated with 3.2 mg/kg of (S)-ketoprofen alone or 3.2 mg/kg of (S)-ketoprofen + 17.8 mg/kg of caffeine diluted in 10% ethanol aqueous solution. Blood samples of 0.2 mL were taken at: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 6.0, 8.0 and 24.0 h, after oral administration of the drug(s), and collected in heparinized tubes. Plasma was separated by centrifugation (3000 rpm, 10 min at 25°C) and immediately frozen at −20°C until assayed, according to the method previously described. At the
conclusion of the study, rats were euthanized with a CO2 overdose to avoid unnecessary suffering.

Plasma samples from the pharmacokinetic study, and a duplicate of three concentrations levels of QC samples, were analyzed together with a standard calibration curve prepared the day of the analysis. Assays were acceptable if the accuracies of the QC samples were within ±15% of the nominal value.

Pharmacokinetic parameters were calculated using WinNonlin Professional 2.0 software. The maximum plasma concentration ($C_{\text{max}}$) was derived from the observed data. The terminal elimination rate constant ($\lambda_z$) was calculated by regression analysis of log-transformed terminal plasma concentration data over time. The half-life ($t_{1/2}$), according to $\lambda_z$, was calculated as $0.693/\lambda_z$. The area under the concentration–time curve (AUC) was calculated by the linear trapezoidal method from zero time to the last sampling time with (S)-ketoprofen concentrations ≥LOQ ($t_L$). The AUC from zero time to infinity ($AUC_{0-\infty}$), was calculated as $AUC_{0-L} + AUC_{L-\infty}$, where $AUC_{L-\infty}$ is the extrapolated part of the AUC from $t_L$ to infinity, which is calculated as $C_{\text{last}}/\lambda_z$, with $C_{\text{last}}$ being the last observed concentration ≥LOQ. The mean residence time (MRT) was calculated as the area under the first-moment curve from zero time to infinity divided by $AUC_{0-\infty}$. The pharmacokinetic parameters were tested using unpaired Student’s t-test after logarithmic transformation. Differences were considered statistically significant if $P < 0.05$.

Results

Method validation

Selectivity

The extraction method allowed adequate separation of the internal standard, (R)- and (S)-ketoprofen from caffeine, and possible endogenous plasma compounds since protonated caffeine and plasma compounds are disposed in the washing process. Good sensitivity and short retention times for drugs were obtained with the HPLC method using the solid-phase extraction procedure proposed. The internal standard, (R)- and (S)-ketoprofen gave well-resolved, sharp peaks with retention times of 3.8, 9.9 and 10.2 min, respectively. Optically active (S)-ketoprofen was used to identify the elution order of ketoprofen enantiomers. No interfering peaks were observed close to the retention times of these compounds with only one-step extraction, after every drug-free rat plasma sample was treated. A typical chromatogram of the studied compounds is shown in Figure 2.

Calibration curves and linearity

When the ratio of each enantiomer to internal standard peak-areas were plotted versus (R)- or (S)-ketoprofen plasma concentrations in the range of 0.25–12.50 μg/mL, a linear relationship was found for both enantiomers ($R^2 > 0.999$). Linear regression equations were $y = 0.42x + 0.06$ for (R)-ketoprofen and $y = 0.45x + 0.03$ for (S)-ketoprofen. Linear regression analysis of the data was significant for the range of the studied concentrations ($P < 0.05$ for both enantiomers) with a CI95% for the intercept from −0.04 to 0.15, and from −0.01 to 0.07 for (R)- and (S)-ketoprofen, respectively.

Figure 2. Chromatograms of (A) drug-free rat plasma, (B) plasma spiked with caffeine (20 μg/mL), (C) plasma spiked with diclofenac (20 μg/mL) as internal standard (IS) and (R, S)-ketoprofen (12.5 μg/mL of each enantiomer) and (D) rat plasma that was taken 1.0 h after oral administration of 3.2 mg/kg of (S)-ketoprofen + 17.8 mg/kg of caffeine.
**Precision, accuracy, LOQ and LOD**

A summary of intra- and interday precision and accuracy of the method is shown in Table I.

The intraday RSD values ranged from 6.0 to 8.5% and 5.5 to 8.4% for (R)- and (S)-ketoprofen, respectively. Interday RSD values were from 11.0 to 14.5%, and 11.0 to 12.5% for (R)- and (S)-ketoprofen, respectively, confirming the precision of the method (RSD <15%). Precision of the method is comparable with a previously published method for enantiospecific HPLC analysis of ketoprofen that used a higher volume of plasma sample (1 mL) and racemic (1-14C)ketoprofen as well as a derivatization step (31).

The intraday RE values ranged from 0.5 to 6.0% and 2.1 to 4.0% for (R)- and (S)-ketoprofen, respectively. Interday accuracy, assessed by the analysis of a set of six replicates of QC samples at three concentrations, repeated during three different days, gave RE values from 3.2 to 12.0% and 1.0 to 11.0% for (R)- and (S)-ketoprofen, respectively. These results demonstrate the accuracy of the method.

Plasma concentrations of (R)- and (S)-ketoprofen can accurately be quantified up to 0.25 μg/mL (LOQ) with RSD and RE values of <20%. It is known that solid-phase extraction provides higher recoveries of the drugs from the biological matrix than liquid–liquid extractions. Consequently, it improves the sensitivity of the method. With the choice of suitable solvents for the conditioning, washing and elution steps, it was demonstrated that the proposed method is more sensitive and efficient than other methods. For instance, Palylyk et al. (32) used a method with higher volume of plasma sample (100 μL), liquid–liquid extractions, an additional derivatization step and reported an LOQ of ketoprofen enantiomers of 0.5 μg/mL. As for Lovlin et al. (35), they reported an LOQ of 0.25 μg/mL, but used 100 μL of plasma samples too. Both methods doubled the sample volume used by us.

The chromatographic background after extraction was clean enough to detect low concentrations of ketoprofen enantiomers. The estimated LOD of 0.15 μg/mL for both enantiomers was verified by injecting a set of QC samples (n = 6) spiked with each enantiomer at this concentration.

**Absolute recovery**

Absolute recoveries, determined by comparing peak-areas from extracted samples with peak-areas of non-extracted standards, were between 108 and 115% with a good precision (RSD <10%), regardless of the concentration studied. The recovery of the method is comparable with other methods that use bigger volume of plasma samples (/7, 16–19/). Two of the advantages of solid-phase extraction procedures are that it allows a better recovery of the analytes from the biological matrix than do liquid–liquid extractions and also allows for multiple samples to be processed at the same time.

**Stability**

Plasma samples containing 1.0 and 10.0 μg/mL were stable after 24.0 h at room temperature, after two freeze–thaw cycles, and after 4 weeks at −20°C (p < 0.05). Stability data are shown in Table II. Additionally, stock solutions of (R, S)-ketoprofen and diclofenac in ethanol, stored at −4°C, were stable for at least 2 weeks. Reconstituted extracted samples in the mobile phase were stable for 24.0 h at room temperature (data not shown). The method showed a good precision with an RSD and RE of <15% in all cases, unrelated to the stability scheme and the evaluated concentrations.

**Robustness**

Small and deliberate changes to the chromatographic conditions used to quantify (R)- and (S)-ketoprofen were used to evaluate the method robustness. Changes made included flow rate, column temperature, batches of columns and different brands of organic solvent. The evaluated changes in the method conditions did not affect the enantiomers resolution significantly, so it confirms the robustness of the proposed method.

**Pharmacokinetic study**

The observed plasma concentration–time curves for (S)-ketoprofen are shown in Figure 3.

No interference peaks attributed to caffeine or endogenous compounds were found during the analysis of samples obtained from the pharmacokinetic study. QC samples in each analytical run were within 15% of the nominal value. (S)-Ketoprofen plasma concentrations found at all sampling times were above the LOQ and within the concentration range of the calibration

**Table I**

<table>
<thead>
<tr>
<th>Enantiomer</th>
<th>Initial (μg/mL)</th>
<th>Final (μg/mL)</th>
<th>RSD (%)</th>
<th>RE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R)-</td>
<td>1.0</td>
<td>9.08</td>
<td>14.5</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>4.80</td>
<td>11.0</td>
<td>4.0</td>
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<tr>
<td></td>
<td>10.0</td>
<td>9.68</td>
<td>11.8</td>
<td>3.2</td>
</tr>
<tr>
<td>(S)-</td>
<td>1.0</td>
<td>9.96</td>
<td>12.5</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>4.95</td>
<td>11.0</td>
<td>1.0</td>
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<tr>
<td></td>
<td>10.0</td>
<td>9.45</td>
<td>11.5</td>
<td>5.5</td>
</tr>
</tbody>
</table>

RSD, relative standard deviation = (standard deviation × 100)/mean; RE, relative error = (nominal – found)/nominal × 100.
Pharmacokinetic parameters of (S)-ketoprofen calculated in this study are close to those reported by Menzel et al. (13). After intravenous administration of 2.5 and 5.0 mg/kg of (S)-ketoprofen to two different groups of rats, the authors reported $AUC_{0-\infty}$ values of $43.4 \pm 16.7$ and $65.9 \pm 23.7$ $\mu$g h/mL, respectively, and $t_1/2$ (half-life) values of 22.0 ± 11.1 and 10.1 ± 5.1 h, respectively. In this study, after oral administration of 3.2 mg/kg of (S)-ketoprofen alone or (S)-ketoprofen + 17.8 mg/kg of caffeine, we found $t_1/2$ values of 17.3 ± 7.30 and 15.40 ± 5.20 h, respectively. In studies reported by Menzel et al. (13), plasma samples were taken from the same animals during a 240-h period. Blood samples must not be taken from the same animal beyond this time, following the Guide for Care and Use of Laboratory Animals (30), and for physiological reasons (low survival rates). The results obtained in our study showed that sampling duration, coupled with the application of the proposed selective analytical micromethod, allowed us to obtain reliable information about (S)-ketoprofen pharmacokinetics after concomitant administration of caffeine to rats.

Conclusion

An easy, sensitive and selective HPLC method for the determination of (S)-ketoprofen without caffeine interference, using 50 $\mu$L of plasma sample, was developed and validated. The method involved a sample preparation with adequate recovery by solid-phase extraction without significant observed matrix effect. The method has been successfully applied to pharmacokinetic study of (S)-ketoprofen after a single oral dose of 3.2 mg/kg of (S)-ketoprofen alone, or in combination with 17.8 mg/kg of caffeine to rats. The method showed to be suitable for 240-h (S)-ketoprofen monitoring in small animals without the need of expensive equipment.

Funding

N.V.G. is a fellow of the National Council for Science and Technology (CONACyT), México (211421). This work includes data from N.V.G.’s Master in Science dissertation, UAM-Xochimilco. Data were obtained during sabbatical break of F.J.L.-M. in UAM-Xochimilco.

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