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Research Article
High-Performance Liquid Chromatography Determination of Meloxicam and Piroxicam with Ultraviolet Detection

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A simple accurate and sensitive high-performance liquid chromatographic method for the determination of meloxicam and piroxicam concentrations in small volume plasma samples has been developed. Following a liquid extraction using chloroform, samples were separated by reversed-phase high-performance liquid chromatography on an XBridge C18 column (4.6 × 250 mm) and quantified using ultraviolet detection at 360 nm. The mobile phase was a mixture of water with glacial acetic acid (pH 3.0) and acetonitrile (50 : 50), with a flow rate of 1.0 mL/min. The standard curve ranged from 5 to 10,000 ng/mL for meloxicam in bearded dragon (Pogona vitticeps) plasma and piroxicam in crane (Grus rubicunda) plasma. Intra- and interassay variability for meloxicam and piroxicam were less than 10% and the average recovery was greater than 90% for both drugs. This method was developed in bearded dragon and crane plasma and should be applicable to any species, making it useful for those investigators dealing with small sample volumes, particularly when conducting pharmacokinetics studies which require multiple sampling from the same animal.

1. Introduction

Meloxicam, 4-hydroxy-2-methyl-N-(5-methyl-1,3-thiazol-2-yl)-1,1-dioxo-1ÂΔ6,2-benzothiazine-3-carboxamide, and piroxicam, 4-hydroxy-2-methyl-1,1-dioxo-N-pyridin-2-yl-1ÂΔ6, 2-benzothiazine-3-carboxamide, are nonsteroidal anti-inflammatory drugs (NSAIDs) with analgesic properties. They are generally used to treat osteoarthritis and rheumatoid arthritis; however, they can be used for other painful conditions such as injuries, cancer surgery, and dental infections. Meloxicam preferentially inhibits the cyclooxygenase-2 (COX-2) enzyme over COX-1. The ability to only inhibit the inflammatory COX proved to be revolutionary for pain management. The introduction of COX-2 preferential NSAIDs has reduced stomach and intestinal side effects. While piroxicam is used to treat painful conditions, it has recently been used in a variety of protocols to treat squamous cell carcinoma and hemangiosarcoma. Meloxicam can be metabolized to as many as four biologically inactive metabolites depending on the species; however, the 5-hydroxy methyl derivative is the most common metabolite. Piroxicam metabolism occurs via a cytochrome P450 2C isoform to multiple biologically inactive metabolites, with 5-hydroxy-piroxicam being the most common.

Nonsteroidal anti-inflammatory drugs are commonly used in large and small veterinary practices for relief of pain, fever, and inflammation. Increasing public awareness of animal welfare will likely continue to make proper use of NSAIDs a priority in the treatment of all domestic animals for painful inflammatory conditions. Therefore knowledge of the pharmacokinetics of NSAIDs in the specific species of interest will be required to provide for the safe and efficacious use of these drugs.

Meloxicam and piroxicam levels have been determined using a number of analytical methods [1–19]. Although mass spectrometry [1, 2, 10, 14] can produce the highest detection sensitivity, it may not be readily available in all laboratories and is expensive. The most economical method is high-performance liquid chromatography with ultraviolet detection (HPLC-UV). In order to obtain a suitable limit of
quantification (LOQ) for the determination of meloxicam and piroxicam in biological samples several different processes have been used. Some of the methods employed include liquid-liquid extractions using diethyl ether [3, 5, 7, 12, 16] or methylene chloride [18, 19], protein precipitation [4, 6, 13, 17], precolumn enrichment [8, 9, 15], and solid phase extraction [11]. In many of the liquid-liquid extraction methods developed, large amounts of organic solvents are needed. Also, sample volumes for all the extraction methods can be large, many requiring from 0.2 to 1 mL of sample. Precolumn enrichment can require the expense of precolumns and the need for column switching. The aim of this paper is to describe a simple, sensitive, and accurate method for extracting meloxicam and piroxicam from small volume plasma samples using HPLC.

2. Material and Methods

2.1. Instrumentation. The chromatography system consisted of a 2695 separation module and a 2487 ultraviolet detector (Waters, Milford, MA). Separation was achieved on a Waters XBridge C18 column (4.6 × 250 mm, 5 μm) preceded by a 5 μm XBridge C18 guard column (3.9 × 20 mm). The mobile phase was an isocratic mixture of A: 990 mL water with 10 mL 85% glacial acetic acid, pH 3.0 with 1 N sodium hydroxide, and B: acetonitrile (50:50). All solutions were filtered through a 0.22 μm filter and degassed before their use. The water was replaced on a daily basis. The flow rate was 1.0 mL/min, and the column and autosampler temperature were ambient which was 23°C. The ultraviolet detector was set at a wavelength of 360 nm.

2.2. Reagents. Meloxicam (Figure 1) was purchased from Toronto Research Chemicals (Ontario, Canada) and was 99% pure. Piroxicam (Figure 1), which was the internal standard (99% purity), was purchased from US Pharmacopeia (Rockville, MD). All reagent grade chemicals and solvents were purchased from Fisher Scientific (Pittsburg, PA). Water (18.2 megaohms) was obtained from a Barnstead Nanopure Infinity (Dubuque, IA) ultrapure water system.

2.3. Preparation of Calibration Standards. Five milligrams each of meloxicam or piroxicam was weighed using a Sartorius Microbalance (Elk Grove, IL) and dissolved in methanol to produce stock concentrations of 100 μg/mL. Dilutions of the meloxicam and piroxicam stock standards were prepared in methanol to produce 0.1, 1, 5, and 10 μg/mL working stock solutions. Standards were aliquoted into 2 mL vials to prevent evaporation and cross contamination. All solutions were protected from light in bottles wrapped in aluminum foil and stored at −20°C. By comparing standard areas over time it was determined that solutions were stable for a minimum of 6 months.

For preparation of calibration standards and quality control samples, appropriate volumes of stock solutions were placed in screw top tubes and evaporated with nitrogen gas and then untreated plasma was added. The final concentrations for the calibration standard curve were 5, 10, 25, 50, 100, 250, 500, 800, 1000, 2500, 5000, and 10,000 ng/mL with quality control standards of 15, 750, 1250, 3000, and 7500 ng/mL. Calibration standards and control samples were treated the same as test samples. The calibration curve was constructed by using the ratio of the peak area of the analyte divided by the peak area of the internal standard versus the concentration. Linearity was assessed by unweighted linear regression analysis. The calibration curve had a correlation coefficient of 0.99 or better. The acceptance criterion for each back-calculated standard and quality control concentration was 15% deviation from the nominal value except for lower limit of quantification (LLOQ), which was set at 20%.

2.4. Sample Extraction. Meloxicam or piroxicam was extracted from bearded dragon (Pogona vitticeps), sandhill crane (Grus canadensis), or sow plasma and sow milk using a liquid extraction. Previously frozen plasma or milk samples were thawed, and 100 μL was placed into a 7 mL screw cap tube. Fifteen microliters of piroxicam for meloxicam extraction (5 μg/mL) was added. After the addition of 100 μL of 1 M hydrochloride and 2 mL of chloroform, tubes were vortex-mixed at a high speed for 60 seconds followed by centrifugation for 20 minutes at 1000×g. The supernatant was removed and placed in a clean 16 × 100 glass test tube and then evaporated with nitrogen. After evaporation, samples were redisolved in 250 μL of the mobile phase and vortex-mixed and the supernatant was placed in total recovery chromatographic vials (Waters, Milford, MA) and then 100 μL injected into the system.

3. Results and Discussion

3.1. Method Development and Optimization. The goal of the study was to develop a simple method that did not require the use of mass spectrometry, column switching, or
large amounts of organic solvents. We found that ultraviolet

detection at 360 nm produced chromatograms with the
largest meloxicam and piroxicam areas compared to those
at 350 nm, 355 nm, and 364 nm. We also tried the extraction
with and without the addition of hydrochloric acid. Without
HCl the average drug recovery was 23% while the addition of
the acid increased recovery to an overall average of
92%.

During method development several organic solutions
and mixtures were tested including acetonitrile, methanol,
acetonitrile:methanol (1:1), hexane, ethyl acetate, methylene
chloride, and chloroform. With the exception of chloroform
the recoveries for the solutions used ranged from 68 to 80%.
There was also decreased peak resolution and/or interference
from plasma components compared to chloroform. Once
chloroform was selected as the extraction solvent the appro-
priate volume was determined. One milliliter produced a
recovery of roughly 75% for both meloxicam and piroxicam
and 3 mL produced the same recovery as 2 mL which was
the volume selected. We also looked at different methods for
mixing the samples and found that vortexing the samples for
sixty seconds produced a greater recovery than rocking for
twenty minutes.

3.2. Analytical Method Validation

3.2.1. Specificity. For specificity testing, untreated crane,
bearded dragon, or sow plasma was prepared in the same
manner as study samples and no endogenous plasma com-
ponents from any species interfered with the elution of the
compounds of interest. Five different blank plasma samples
from adult male and female bearded dragons or cranes were
used in the prevalidation process and a blank sample from each
animal was included in the analysis. Five different blank plasma samples from adult sows were used in the
prevalidation process and a blank sample from each animal
was also analyzed. Meloxicam and piroxicam metabolites
are biologically inactive so no effort was made to analyze
these compounds in any species. Figure 2 illustrates chro-
matograms of a (a) blank bearded dragon sample, (b) a
250 ng/mL spiked plasma standard, (c) a plasma sample
from a bearded dragon after intramuscular administration of
0.4 mg/kg meloxicam, (d) a blank sandhill crane sample, (e)
an 800 ng/mL spiked plasma standard, (f) a plasma sample
from a sandhill crane after oral administration of 0.5 mg/kg
piroxicam, (g) a blank sow milk sample, (h) a 250 ng/mL
spiked milk standard, and (i) a milk sample from a sow after
intramuscular administration with 0.8 mg/kg meloxicam.
The retention times in both bearded dragon and sandhill


crane plasma were 73 and 5.9 minutes for meloxicam and
piroxicam, respectively. The retention times in milk were 7.6
and 5.9 minutes.

For milk specificity testing, untreated sow milk was
prepared in the same manner as study samples and no
endogenous milk components interfered with the elution of the
compounds of interest. Five different blank milk samples
from adult sows were used in the prevalidation process and a
blank sample from each animal was also analyzed.

3.2.2. Linearity, Accuracy, and Precision. The plasma peak
ratio (area of meloxicam or piroxicam divided by the internal
standard area) was plotted and produced a linear curve for
the concentration range used (5–10,000 ng/mL) for each drug
with a correlation coefficient of 0.995 to 0.999. The mean
slope, intercept, and r² values are reported in Tables 1, 2, and 3.
Intra- and inter-day assay relative standard deviation (RSD)
for plasma spiked with specific concentrations of meloxicam
or piroxicam were used to determine accuracy and precision
which ranged from 11% to 11.3%. The precision was found to
be below the set ±15% for all quality control samples. The use
of an internal standard corrects for the intra- and interday
assay variability in the assay.

3.2.3. Recovery and Limit of Quantification (LLOQ). The
recovery (Tables 4, 5, and 6) of meloxicam and piroxicam
from spiked plasma and milk was determined by comparing
areas of extracted plasma or milk samples to those of directly
injected analytes without undergoing extraction at concen-
trations of 15, 750, 1250, 3000, and 7500 ng/mL. The average
plasma recovery ranged from 91% to 97% for meloxicam and
from 88% to 94% for piroxicam. The average recovery from
milk ranged from 88% to 95% for meloxicam. The lower
limit of quantification (LLOQ) was 5 ng/mL for both drugs
in both plasma and milk matrix, which represents a peak
approximately five times the baseline noise. The LLOQ is
more than adequate for use in pharmacokinetic studies. If a
lower LLOQ is needed sample size could be increased.

3.2.4. Stability. Testing of the short term stability of the
quality control standards for both milk and plasma indicated
there was less than a 1% loss of either drug after 24 hours
in the autosampler and a 1% loss of both drugs after 24
hours in the refrigerator at 4°C. Therefore, if there was power
or equipment failure samples could be reanalyzed. Samples
in our studies were stored at ~80°C, thawed one time, and
analyzed; however, there was no loss of drug after 2 freeze-
thaw cycles. There was also no loss of drug after two weeks
of storage at ~80°C which is one week longer than study samples
were stored for analysis.

3.3. Application of Method

3.3.1. Method Application. Figure 3 is a representative


centration-time profile from a meloxicam pharmacokinetic
study conducted in bearded dragons (Pogona vitticeps) after
receiving a 0.4 mg/kg intramuscular dose of meloxicam.
The $T_{\text{max}}$, $C_{\text{max}}$, and half-life for this animal were 0.75 h,
3953 ng/mL, and 4.9 h, respectively. Figure 4 is a represent-
ative concentration-time profile from a piroxicam pharma-
cookinetic study conducted in sandhill cranes (Grus canadens-
is) after receiving a 0.5 mg/kg oral dose of piroxicam. The
$T_{\text{max}}$, $C_{\text{max}}$, and half-life for this animal were 1 h, 4840 ng/mL,
and 73 h, respectively.
Figure 2: Chromatograms for meloxicam and piroxicam. (a) Blank bearded dragon plasma sample, (b) a 250 ng/mL spiked plasma standard, (c) a plasma sample from a bearded dragon after intramuscular administration of 0.4 mg/kg meloxicam, (d) a blank sandhill crane plasma sample, (e) an 800 ng/mL spiked plasma standard, (f) a plasma sample from a sandhill crane after oral administration of 0.5 mg/kg piroxicam, (g) a blank sow milk sample, (h) a 250 ng/mL spiked milk standard, and (i) a milk sample from a sow dosed with 0.8 mg/kg meloxicam.
### Table 1: Intra-assay accuracy, precision and assay linearity for meloxicam in plasma.

<table>
<thead>
<tr>
<th>Concentration added (ng/mL)</th>
<th>Concentration measured (ng/mL) bearded dragon (Mean ± S.D.)</th>
<th>R.S.D. (%)</th>
<th>Concentration measured (ng/mL) pig (Mean ± S.D.)</th>
<th>R.S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>15 ± 0.71</td>
<td>4.6</td>
<td>15 ± 0.21</td>
<td>1.4</td>
</tr>
<tr>
<td>750</td>
<td>708 ± 28</td>
<td>4.0</td>
<td>757 ± 69</td>
<td>9.1</td>
</tr>
<tr>
<td>1250</td>
<td>1156 ± 46</td>
<td>4.0</td>
<td>1149 ± 2.8</td>
<td>0.25</td>
</tr>
<tr>
<td>3000</td>
<td>2982 ± 155</td>
<td>5.1</td>
<td>2990 ± 160</td>
<td>5.3</td>
</tr>
<tr>
<td>7500</td>
<td>7528 ± 758</td>
<td>10.0</td>
<td>7300 ± 680</td>
<td>9.3</td>
</tr>
</tbody>
</table>

Assay linearity (n = 5)

<table>
<thead>
<tr>
<th>Mean ± S.D.</th>
<th>R.S.D. (%)</th>
<th>Mean ± S.D.</th>
<th>R.S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( Y )-Intercept</td>
<td>0.0037 ± 0.0004</td>
<td>11.3</td>
<td>0.0033 ± 0.0003</td>
</tr>
<tr>
<td>Slope</td>
<td>0.0020 ± 0.0002</td>
<td>12.3</td>
<td>0.0021 ± 0.0002</td>
</tr>
<tr>
<td>( r^2 )</td>
<td>0.995 ± 0.0028</td>
<td>0.28</td>
<td>0.994 ± 0.0038</td>
</tr>
</tbody>
</table>

S.D.: standard deviation; n: number of samples; RSD: relative standard deviation.

### Table 2: Intra-assay accuracy, precision and assay linearity for piroxicam in plasma.

<table>
<thead>
<tr>
<th>Concentration added (ng/mL)</th>
<th>Concentration measured (ng/mL) (Mean ± S.D.)</th>
<th>R.S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>21 ± 0.41</td>
<td>2.0</td>
</tr>
<tr>
<td>750</td>
<td>774 ± 27</td>
<td>3.5</td>
</tr>
<tr>
<td>1250</td>
<td>1293 ± 44</td>
<td>3.4</td>
</tr>
<tr>
<td>3000</td>
<td>3006 ± 69</td>
<td>2.0</td>
</tr>
<tr>
<td>7500</td>
<td>7517 ± 399</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Assay linearity (n = 5)

<table>
<thead>
<tr>
<th>Mean ± S.D.</th>
<th>R.S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( Y )-Intercept</td>
<td>−0.0106 ± 0.0004</td>
</tr>
<tr>
<td>Slope</td>
<td>0.0012 ± 0.000018</td>
</tr>
<tr>
<td>( r^2 )</td>
<td>0.997 ± 0.003</td>
</tr>
</tbody>
</table>

S.D.: standard deviation; n: number of samples; RSD: relative standard deviation.

### Table 3: Intra-assay accuracy, precision and assay linearity for meloxicam in milk.

<table>
<thead>
<tr>
<th>Concentration added (ng/mL)</th>
<th>Concentration measured (ng/mL) (Mean ± S.D.)</th>
<th>R.S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>15 ± 0.41</td>
<td>2.7</td>
</tr>
<tr>
<td>750</td>
<td>727 ± 27</td>
<td>3.7</td>
</tr>
<tr>
<td>1250</td>
<td>1149 ± 30</td>
<td>2.6</td>
</tr>
<tr>
<td>3000</td>
<td>3000 ± 69</td>
<td>2.3</td>
</tr>
<tr>
<td>7500</td>
<td>7450 ± 399</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Assay linearity (n = 5)

<table>
<thead>
<tr>
<th>Mean ± S.D.</th>
<th>R.S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( Y )-Intercept</td>
<td>−0.0079 ± 0.0004</td>
</tr>
<tr>
<td>Slope</td>
<td>0.0017 ± 0.000013</td>
</tr>
<tr>
<td>( r^2 )</td>
<td>0.999 ± 0.003</td>
</tr>
</tbody>
</table>

S.D.: standard deviation; n: number of samples; RSD: relative standard deviation.
**Table 4:** Inter-assay variability and recovery for meloxicam in plasma and (n = 5).

<table>
<thead>
<tr>
<th>Concentration added (ng/mL)</th>
<th>Concentration measured bearded dragon (ng/mL) (Mean ± S.D.)</th>
<th>R.S.D. (%)</th>
<th>Recovery (%)</th>
<th>Concentration measured pig (ng/mL) (Mean ± S.D.)</th>
<th>R.S.D. (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>14 ± 1</td>
<td>7.1</td>
<td>97</td>
<td>15 ± 0.4</td>
<td>3.0</td>
<td>99</td>
</tr>
<tr>
<td>750</td>
<td>743 ± 15</td>
<td>2.1</td>
<td>91</td>
<td>746 ± 12</td>
<td>1.5</td>
<td>98</td>
</tr>
<tr>
<td>1250</td>
<td>1220 ± 73</td>
<td>6.0</td>
<td>94</td>
<td>1227 ± 21</td>
<td>1.7</td>
<td>95</td>
</tr>
<tr>
<td>3000</td>
<td>3011 ± 128</td>
<td>4.3</td>
<td>95</td>
<td>2970 ± 32</td>
<td>1.1</td>
<td>95</td>
</tr>
<tr>
<td>7500</td>
<td>7467 ± 562</td>
<td>7.5</td>
<td>95</td>
<td>7466 ± 330</td>
<td>4.4</td>
<td>95</td>
</tr>
</tbody>
</table>

SD: standard deviation; n: number of days; RSD: relative standard deviation.

**Table 5:** Inter-assay variability and recovery for piroxicam in plasma and (n = 5).

<table>
<thead>
<tr>
<th>Concentration added (ng/mL)</th>
<th>Concentration measured (ng/mL) (Mean ± S.D.)</th>
<th>R.S.D. (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>22 ± 2</td>
<td>9.9</td>
<td>88</td>
</tr>
<tr>
<td>750</td>
<td>746 ± 56</td>
<td>7.4</td>
<td>94</td>
</tr>
<tr>
<td>1250</td>
<td>1255 ± 142</td>
<td>11.3</td>
<td>88</td>
</tr>
<tr>
<td>3000</td>
<td>2976 ± 147</td>
<td>4.9</td>
<td>89</td>
</tr>
<tr>
<td>7500</td>
<td>7500 ± 419</td>
<td>5.6</td>
<td>88</td>
</tr>
</tbody>
</table>

SD: standard deviation; n: number of days; RSD: relative standard deviation.

**Table 6:** Inter-assay variability and recovery for meloxicam in milk (n = 5).

<table>
<thead>
<tr>
<th>Concentration added (ng/mL)</th>
<th>Concentration measured (ng/mL) (Mean ± S.D.)</th>
<th>R.S.D. (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>17 ± 2</td>
<td>10.8</td>
<td>94</td>
</tr>
<tr>
<td>750</td>
<td>730 ± 68</td>
<td>9.2</td>
<td>91</td>
</tr>
<tr>
<td>1250</td>
<td>1233 ± 78</td>
<td>6.3</td>
<td>90</td>
</tr>
<tr>
<td>3000</td>
<td>2976 ± 131</td>
<td>4.4</td>
<td>95</td>
</tr>
<tr>
<td>7500</td>
<td>7500 ± 330</td>
<td>4.4</td>
<td>88</td>
</tr>
</tbody>
</table>

SD: standard deviation; n: number of days; RSD: relative standard deviation.

**4. Conclusion**

In conclusion, this HPLC method quantifies meloxicam or piroxicam from plasma by combining a liquid extraction procedure with ultraviolet detection. This analytical procedure was authenticated in terms of recovery, linearity, LLOQ, precision, and accuracy. The limit of quantification and recovery are more than adequate for use in pharmacokinetic studies. The results of this study indicate that this HPLC procedure is a reproducible method that provides consistent quantification of meloxicam or piroxicam in plasma from different species.

It is a simple method that eliminates the need for mass spectrometry, column switching, and large volumes of organic solvents. It allows numerous samples to be processed by a single technician. This method has been used successfully to determine meloxicam and piroxicam concentrations in plasma and milk samples at this institution. This method utilizes a small sample size of 100 μL making it potentially useful for small dogs, cats, or other small animals. It could be particularly useful to investigators when conducting pharmacokinetic studies in small animals which require multiple sampling from the same animal.
Conflict of Interests
The authors declare that there is no conflict of interests regarding the publication of this paper.

References